NEUROGLIOGENESIS AND VISUAL SYSTEM DEVELOPMENT IN *DROSOPHILA*: GENETIC / GENOMIC ANALYSIS OF THE *GLIAL CELLS MISSING* AND *EGGHEAD* GENES

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

Yun Fan
aus Jiangxi (CHINA)

Basel 2005

Zoologisches Institut der Universität Basel
Pharmazentrum / Biozentrum
Klingelbergstrasse 50
CH-4056 Basel

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von

Prof. Dr. Heinrich Reichert (Fakultätsverantwortlicher)

Prof. Dr. Karl-Friedrich Fischbach (Korreferent)

Basel, den 5. Juli 2005

Dekan Prof. Dr. Hans-Jakob Wirz

CONTENTS

1. SUMMARY

Complex nervous systems are made up by two major cell types, neuronal and glial cell types. A general observation that has been made by lineage analysis of neurogliogenesis in vertebrates and invertebrates is that neurons and glia often share common progenitors. Therefore an interesting question in cellular neurobiology is how neuronal versus glial cell fate is established. In *Drosophila, glial cells missing (gcm)* is a key control gene of gliogenesis. In *gcm* mutants, presumptive glial cells are transformed into neurons and, conversely, when *gcm* is ectopically misexpressed, presumptive neurons become glia. Since *gcm* encodes a transcription factor, it is proposed that a set of downstream genes are regulated by GCM that in turn execute the glial differentiation program.

In the first set of experiments carried out in this thesis, genome-wide oligonucleotide arrays were used to identify *gcm* downstream genes in a comprehensive manner. Differential gene expression was analyzed in wild type embryos and compared to embryos in which *gcm* was misexpressed throughout the neuroectoderm. We found hundreds of genes that were differentially expressed following *gcm* misexpression. They thus are potentially involved in aspects of glial development. This study is one of the first genome-wide analyses of gene expression events downstream of a key developmental transcription factor and represents a novel level of insight into the repertoire of genes that initiate and maintain cell fate choices in the development of the central nervous system.

Microarrays are powerful and efficient tools to quantify and compare gene expression on a large scale. However, as with all large-scale experiments, microarray experiments can be influenced by inherent biological factors. In vivo analysis suggests a low level of validation of the initial microarray data we obtained for *gcm* downstream genes. One of the main reasons accounting for this low verification rate appears to be the complexity of the tissue used for the microarray experiments. In the second microarray analysis of *gcm* gene action in neurogliogenesis performed in this thesis, tissue heterogeneity was reduced by using the technique of magnetic cell separation (MACS) to isolate neuroectoderm cells from *Drosophila* embryos. Validation studies by in situ hybridization of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed high rates of verification. This suggests that reduction

of cell heterogeneity increases the ability of microarrays to reveal differential gene expression in the developing nervous system.

The subsequent major part of this thesis addresses the role of the *egghead* (*egh*) gene, a putative *gcm* downstream target that was identified by microarray analyses, in visual system development of *Drosophila*. It is known that the correct targeting of photoreceptor neurons (R-cells) in the developing *Drosophila* visual system requires multiple guidance systems in the eye-brain complex as well as the precise organization of the target area. Although the molecular mechanisms that underlie the targeting of R-cell axons have been studied intensively in the photoreceptor neurons of the developing eye, and to a lesser degree in the developing lamina and medulla, little is known about the possible role of the lobula complex which transiently abuts the lamina and medulla in the developing larval brain. In our study, we find that the *egh* gene, encoding a glycosyltransferase, is required for a compartment boundary between lamina glia and lobula cortex, which is necessary for appropriate retinal innervation of the lamina. In the absence of *egh*, perturbation of sheath-like glial processes occurs at the boundary region delimiting lamina glia and lobula cortex, and inappropriate invasion of lobula cortex cells across the boundary region disrupts the pattern of lamina glia resulting in inappropriate R1-R6 axonal projections. Further genetic analysis involving mosaics demonstrates that the requirement of *egh* is restricted to the lobula complex primordium. This study thus uncovers a novel role of *egh* gene function in the developing *Drosophila* visual system and underscores the unexpected role of the lamina/lobula compartment boundary in R1-R6 axon targeting.

2. INTRODUCTION

2.1. Embryonic development of *Drosophila* central nervous system

The insect central nervous system (CNS) in terms of anatomical organization differs strikingly from the vertebrate CNS. Insects, like other protostome gastroneuralia, have an anterior brain and a ventral ganlionic chain, the ventral nerve cord (VNC), while vertebrates, like other deuterostome notoneuralia, have a complex anterior brain and a dorsal nerve cord. Contrasting with these morphological differences are remarkable similarities in the genetic control mechanisms that operate during CNS development in protostomes and deuterostomes. For example, in insects and vertebrates, comparable molecular genetic pathways provide positional information for patterning the developing brain along the dorsoventral and anteroposterior body axes (Reichert and Simeone, 2001; Lichtneckert and Reichert, 2005). Additionally, comparable molecular genetic pathways act during neurogenesis and gliogenesis in both phyla in order to promote cell fate specification (Cornell and Ohlen, 2000; Bertrand et al., 2002). Thus, the relatively simple CNS of the *Drosophila* embryo offers a key to the understanding of the mechanisms that generate and pattern complex nervous systems.

The *Drosophila* CNS develops from a bilateral neuroectoderm that lies on either side of a narrow strip of ventral midline cells and can be subdivided into the brain and the VNC. Studies on the *Drosophila* VNC have been crucial for elucidating the molecular genetic mechanisms that control nervous system development (Skeath and Thor, 2003; Fig.1). In early stages of embryonic development, single neuroectodermal cells delaminate from the surface epithelium in a fixed pattern, and move into the interior of the embryo to form neural precursor cells called neuroblasts (NBs). In each hemisegment of the VNC, about 30 neuroblasts are generated in approximately five successive waves along the anterior-posterior (AP) and dorsoventral (DV) axes in a stereotyped and spatiotemporal pattern. Subsequently, each neuroblast expresses a characteristic combination of genes and contributes a stereotyped family of neurons and glia to the CNS. At the end of embryonic neurogenesis, each hemisegment consists of ~350 neurons and ~30 glia (Doe, 1992; Broadus et al., 1995; Campos-Ortega and Hartenstein, 1997). After that, most neuroblasts stop dividing and remain quiescent in the neurogenic regions until larval stages (Truman and Bate, 1988; Datta, 1995; Maurange and Gould, 2005).

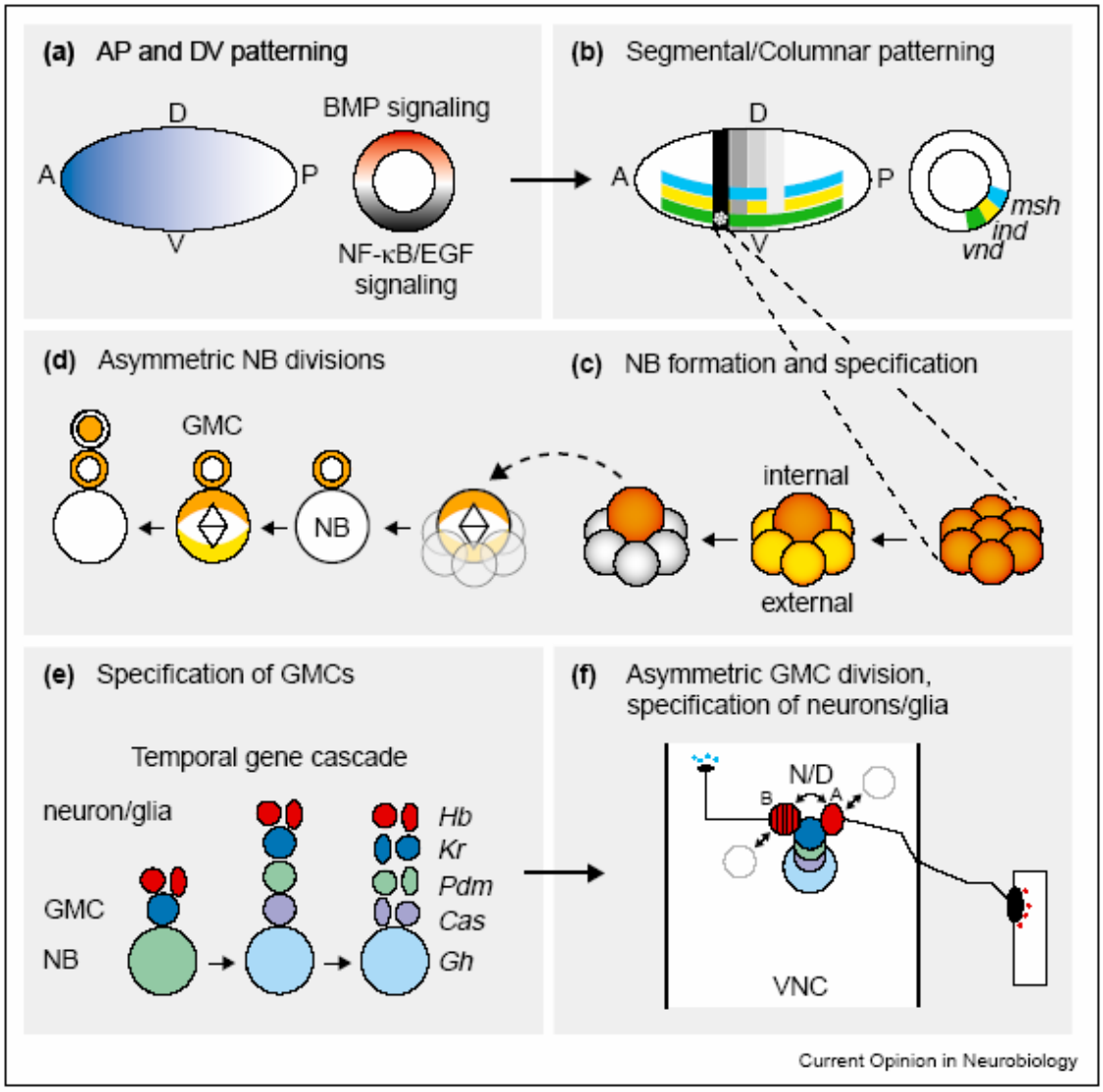

Fig.1. Development of the *Drosophila* VNC. (a) In the early embryo, complex cascades of patterning genes act in gradients along the AP and DV axes. (b) These events lead to the expression of segment-polarity (black-gray) and columnar (green-yellow-blue) genes in discrete stripes in each segment and along the neuroectoderm, respectively. The segment-polarity and columnar genes subdivide the neuroectoderm, such that each neural equivalence group (white dots) expresses a unique combination of regulatory genes. For clarity, only 1 of the 14 hemisegments of the VNC is shown. (c) In each neural equivalence group, expression of the *ac/sc* genes (red) is initially uniform. Lateral inhibition mediated by Notch and Delta generates one NB by suppressing the expression of *ac/sc* genes in surrounding cells. The NB delaminates and moves from the external surface of the ectoderm to the internally forming VNC. (d) The newly delaminated NB begins a series of asymmetric divisions, controlled by basal (yellow) and apical (orange) protein complexes. The key step in this process involves the segregation of Prospero (orange) into the GMC, where it localizes transiently to the cell cortex. Prospero then quickly translocates to the nucleus, where it represses the expression of cell-cycle genes, thereby limiting the proliferative potential of GMCs. (e) The temporal expression of *Hb* (red) *Kr* (blue) *Pdm* (green) *Cas* (purple) and *Gh* (light blue) leads to diversification of the GMCs generated by each NB. Most, if not all, GMC divisions are, in turn, asymmetric (oval versus circle) and generate postmitotic sibling cells of different types. (f) Asymmetric GMC divisions are governed by antagonistic interactions between the Notch pathway and Numb. Surrounding cells (light gray) signal through the Notch pathway for both siblings to acquire the Notch-dependents 'A-cell' fate. The asymmetric distribution of Numb (hatched box) into one sibling blocks Notch signaling in this cell and promotes the 'B-cell' fate. Active Notch signaling in the other cell promotes the A-cell fate. Thus, the combined effects of the NB identity genes, as determined by the segment-polarity and columnar genes (b), the temporal gene cascade (e) and the asymmetric division of GMCs leads to the specification of unique types of neuronal and glial cells. By the end of embryogenesis, these processes result in the formation and specification of 60 glia and around 700 neurons in each segment of the VNC, which have many different cell morphologies and utilize many different neurotransmitters (red and blue dots) (adapted from Skeath and Thor, 2003).

2.1.1. Patterning the neuroectoderm

Extensive molecular genetic studies have identified genetic cascades that pattern the ventral neuroectoderm along its AP and DV axes (Skeath and Thor, 2003; Fig.1a and 1b). On the one hand, the sequential action of the maternal AP coordinate, gap and pair-rule genes, defines the location of each AP stripe of segment-polarity gene expression in a segment (Akam, 1987). The segment-polarity genes, such as *wingless*, *hedgehog*, *gooseberry*, and *engrailed*, in turn are expressed in a segmentally reiterated manner along the AP axis and enable neuroblasts that form in different AP rows to acquire different fates (Bhat, 1999). Detailed examination of the role of many of the segment polarity genes during neurogenesis indicates that they are required for establishing AP row identity within the neuroectoderm and neuroblasts (Chu-LaGraff and Doe, 1993; Zhang et al., 1994; Skeath et al., 1995; Bhat, 1996; Matsuzaki and Saigo, 1996; Bhat and Schedl, 1997; McDonald and Doe, 1997). For instance, *gooseberry* is expressed in row 5 neuroectoderm. Embryos lacking *gooseberry* function have a transformation of row 5 into row 3 neuroectoderm and neuroblast identity, whereas misexpression of *gooseberry* results in the converse row 3 to row 5 transformation (Zhang et al., 1994; Skeath et al., 1995). Similarly, *wingless* encodes a protein secreted from row 5 and required for specifying the fate of the adjacent rows 4 and 6 neuroectoderm and neuroblasts (Chu-LaGraff and Doe, 1993).

On the other hand, three signaling pathways, Dorsal (Dl), Decapentaplegic (Dpp) and Epidermal growth factor receptor (Egfr) signaling, determine the DV extent of the neuroectoderm: Dl signaling is required for ventral mesoderm and neuroectoderm formation, Dpp signaling defines the dorsal border of the neurogenic region and Egfr signaling is crucial for ventral and intermediate neuroectoderm specification (Cornell and Von Ohlen, 2000). Maternally contributed Dl protein is a member of the Rel/NF-κB family transcription factors, which is initially distributed throughout the cytoplasm of developing oocytes but is transported into nuclei shortly after fertilization (Steward, 1987). In early embryos, the Dl protein is selectively transported into ventral nuclei in a graded fashion such that the highest levels of Dl protein are found in the most ventral nuclei. This gradient initiates the differentiation of mesoderm, neuroectoderm, and dorsal ectoderm, and help prepattern the mesoderm and neuroectoderm. High nuclear concentrations of Dl at the ventral side of the embryo induce expression of mesodermal genes *twist* and *snail,* which in turn repress neuroectoderm formation. On the lateral sides, lower levels of nuclear Dl give rise to neuroectoderm and are required to activate neural gene expression directly or indirectly. Dl

may, in part, induce neural genes indirectly via activation of inhibitors of Dpp signaling; such inhibitors include *short gastrulation* (*sog*), an extracellular antagonist of Dpp, and *brinker* (*brk*), a transcriptional repressor of Dpp-activated genes (Stathopoulos and Levine, 2002). The *dpp* gene encodes a member of the transforming growth factor beta (TGF-β) superfamily and is expressed in tissue dorsal to the neuroectoderm (prospective dorsal epidermis). Dpp expression defines the dorsal border of the presumptive neuroectoderm and also has an essential role in establishing the dorsal embryonic tissue including dorsal ectoderm and the extra-embryonic tissue, called the amnioserosa. The *dpp* loss-of-function mutant phenotype shows a marked expansion of the neurogenic ectoderm at the expense of dorsal structures such as the amnioserosa. In contrast, when the *dpp* gene is misexpressed ventrally, it can induce dorsal structures and inhibit neurogenic tissue formation (Ferguson and Anderson, 1992; Wharton et al., 1993). Hence, the Dpp pathway defines the dorsal limit of the CNS and may contribute to its patterning.

Together with Dl and Dpp, the Egfr signaling pathway controls the further subdivision of the neuroectoderm into three DV domains or columns which are characterized by the expression of one of the three homeodomain-containing columnar genes: *ventral nervous system defective* (*vnd*), *intermediate neuroblast defective* (*ind*) and *muscle segment homeobox* (*msh*). *vnd* is expressed in the ventral column, *ind* is expressed in the intermediate column and *msh* is expressed in the dorsal column. The *vnd* domain is established by Dl and maintained by Egfr signaling. *ind* expression requires both Dl and Egfr signaling. The *msh* domain is defined by repression: it occurs only where Dpp, Vnd and Ind activity are low (Von Ohlen and Doe, 2000). There is also evidence that Egfr signaling controls aspects of ventral and intermediate column identity and may position the border between the intermediate and dorsal columns. In *egfr* mutant embryos, dorsal column genes are expressed in the intermediate neuroectoderm and intermediate neuroblasts fail to form (Skeath, 1998; Yagi et al., 1998). Moreover, genetic data suggest the existence of a hierarchical cascade of transcriptional repression amongst the DV columnar genes: *vnd* represses *ind* in the ventral column and *ind* represses *msh* in the intermediate column (McDonald et al., 1998; Weiss et al., 1998).

In consequence of these AP and DV patterning events, the neuroectoderm of each hemisegment is subdivided into a checkerboard pattern of neural equivalence groups, and each equivalence group contains a unique combination of segment-polarity and columnar gene activities (Skeath and Thor, 2003). Each unique combination of gene activities then

induces and regulates the expression of a distinct set of genes which acts to control the identity of the neuroblast that segregates from the neuroectoderm.

2.1.2. Specification and division of neuroblasts

Neuroblast formation is regulated by two important classes of genes: proneural genes and neurogenic genes. Proneural genes, which encode a family of basic helix-loop-helix (bHLH) transcription factors, are necessary and sufficient to initiate neural differentiation in the neuroectoderm. Molecular studies have identified four proneural genes belonging to the *acheate-scute* complex (*asc*), namely *acheate* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) (Ghysen and Dambly-Chaudiere, 1989; Campuzano and Modolell, 1992). A second family of proneural genes, *atonal* (*ato*) and its related genes *amos* and *cato*, were isolated more recently. They also belong to the same class of bHLH transcriptional factors (Bertrand et al., 2002). *asc* and *ato* families account for all proneural activity in the PNS, but not in the CNS. This suggests that there are further proneural genes which might diverge in structure from those already identified. Proneural genes are expressed in 4-6 cell clusters (equivalence groups) at specific positions within the neuroectoderm. In each equivalence group, a single cell is selected to acquire a neural progenitor or so-called neuroblast fate (Skeath and Thor, 2003; Fig.1c). This is achieved through activation of the Delta/Notch signaling pathway, in a process termed "lateral inhibition", and is based on a molecular regulatory loop between adjacent cells. *Notch* and *Delta* are neurogenic genes and encode transmembrane proteins (Fleming, 1998). Expression of the Notch ligand, Delta, in the future neuroblasts activates the Notch signaling cascade in neighboring cells, resulting in the expression of repressors such as the *E(spl)* genes that, in turn, directly downregulate proneural gene expression. As a result, proneural gene expression is restricted to single cells that enter a neural-differentiation pathway (Skeath and Carroll, 1994; Artavanis-Tsakonas et al., 1999).

After delaminating basally from the surface epithelium, each Neuroblast undergoes an apical/basal (A/B) oriented asymmetric cell division, giving rise to a small basal ganglion mother cells (GMC) and a larger apical neuroblast (Skeath and Thor, 2003; Fig.1d). Neuroblasts continue to divide in this manner, while each GMC divides only once more, asymmetrically, to generate neurons and glial cells. The orientation of the first division of a neuroblast lineage is initiated by inheritance of an evolutionarily conserved protein cassette consisting of Bazooka (Baz), DaPKC and DmPar6, which act to mediate polarity in epithelia as well as other developmental contexts. In delaminating interphase neuroblasts this protein

cassette, which is apically localized, is joined by Inscuteable (Insc), Partner of Inscuteable (Pins) and Gαi to form an apical protein complex that establishes neuroblast A/B polarity. Once the A/B polarity is established, the mitotic spindle is set up and oriented along the axis of polarity. Meanwhile the cell fate determinants, Numb, Prospero, *prospero* RNA and adaptor molecules that facilitate their localization, Miranda, Partner of Numb and Staufen, form basal cortical crescents and segregate preferentially to the basal daughter (GMC) (Chia and Yang, 2002; Bardin et al., 2004; Betschinger and Knoblich, 2004; Roegiers and Jan, 2004). Hence, during the first asymmetric cell division of a newly delaminated neuroblast, the apical protein complex coordinates mitotic spindle orientation with the basal localization of cell fate determinants. In contrast, the molecular mechanism of subsequent neuroblast and GMC divisions in the CNS is currently unclear. However, the study on the sensory organ lineage provides a possible mechanism by which the orientations of later divisions might be specified by those of earlier divisions of the same lineage (Le Borgne et al., 2002).

2.1.3. Specification and division of ganglion mother cells

Nearly every GMC is thought to acquire a unique fate. A temporal transcription factor cascade has been found in most of neuroblast lineages to enable GMCs born at different times in a lineage to acquire distinct fates (Skeath and Thor, 2003; Fig.1e). So far, five members of this temporal cascade have been identified in the early embryo and they are expressed in the sequential order: Hunchback (Hb)→ Kruppel(Kr)→ POU domain proteins (Pdm)→ Castor (Cas)→ Grainyhead (Gh or Grh). Notably, in some neuroblast lineages, only subsets of these transcription factors are present. Moreover, the putative Cas→ Gh transition has not been documented within the developing embryo and it remains to be seen whether Gh specifies late GMC identity (Kambadur et al., 1998; Brody and Odenwald, 2000; Isshiki et al., 2001; Novotny et al., 2002; Pearson and Doe, 2003). This orderly progression of gene activity results in a layered pattern of gene expression in the neurons and glia produced by each neuroblast. Hb-possitive neurons are located at the basal edge of the VNC and Gh-positive neurons are located at the apical edge, with Kr-, Pdm- and Cas-positive neurons sandwiched in between. Loss- and gain-of-function experiments suggest extensive crossregulation among these transcription factors such that the earlier expressed transcription factor activates the next gene in the pathway and concomitantly represses the “next plus one” gene. In addition, it is likely that other inputs participate in temporal regulation of gene expression in neuroblasts and these are tightly linked with cell cycle regulation (Brody and Odenwald, 2002; Novotny et al., 2002; Pearson and Doe, 2003).

Once formed, each GMC divides asymmetrically to produce two postmitotic neurons and/or glia that acquire distinct fates (Skeath and Thor, 2003; Fig.1f). Studies indicate that the *Notch* signaling pathway acts in opposition to that of *numb* to enable most, if not all, GMCs to divide asymmetrically to generate sibling neurons with distinct fates. The asymmetric distribution of Numb into one daughter cell blocks Notch signaling in this cell and promotes its terminal cell fate, while the other sibling acquires the different and Notch-dependent cell fate. However, the mechanism by which Numb inhibits Notch is not yet clear although several studies have suggested two models to explain how Numb functions (Berdnik et al., 2002; Justice et al., 2003; Le Borgne et al., 2003; O'Connor-Giles and Skeath, 2003; Roegiers and Jan, 2004). In one model, Numb downregulates Notch signaling by endocytosis of the Notch receptor mediated by α-Adaptin, and perhaps Lethal giant larvae (Lgl). In another model, Numb downregulates Notch signaling by inhibiting the membrane interaction of Sanpodo, but the role of α-Adaptin and Lgl in Sanpodo membrane association has not yet been explored.

2.2. Gliogenesis in *Drosophila*: *glial cells missing* (*gcm*) gene action

Complex nervous systems are made up by two major cell types, neuronal and glial cell types. As described above, during the development of *Drosophila* CNS, neural progenitors delaminate from the ectoderm and cycle through a series of asymmetric divisions, producing a secondary precursor called a ganglion mother cell (GMC) with each event. Each GMC then passes through a single division to yield differentiated neurons and/or glia. One population of CNS progenitors, neuroblasts, gives rise to only neurons, whereas glial producing progenitors come in two forms: glioblasts, which give rise to only glial cells, and neuroglioblasts, which produce mixed glial/neuronal lineages. Moreover, neuroglioblasts can be further subdivided into at least two types: type 1 neuroglioblasts produce a glioblast and a neuroblast after the first division, and type 2 neuroglioblasts generate a series of GMCs that divide once to yield either two sibling neurons or a neuron/glia sibling pair (Udolph et al., 2001; Jones, 2005). For simplicity, CNS progenitors are often collectively called neuroblasts (NBs). In the peripheral nervous system (PNS), sensory organ precursor cells (SOPs) delaminate from the ectoderm and undergo a series of cell divisions that generate specific types of neurons, glia, and other support cells. Thus, NBs and SOPs generate unique, reproducible, stereotypic pattern of neuronal and glial progeny (Bossing et al., 1996; Schmidt et al., 1997; Schimid et al., 1999;

Roegiers et al., 2001). In addition, midline glial cells in the CNS are generated separately from mesectoderm cells (see below).

2.2.1. Categories of glia in the embryonic central nervous system

Whereas neurons send out long processes to form the intricate neuronal network that collects, integrates and transmits information, numerous functions have been attributed to glial cells ranging from important functions during the development of the complex neuronal network to electrical insulation of mature neurons (Klambt, 2001). During embryonic development, most axon tracts develop in close association with different glial cells. For example, the midline glial cells are required for the separation of commissures and the correct organization of axon fascicles within the commissures (Hummel et al., 1999a,b). The longitudinal glial cells are involved in growth cone guidance and direct the fasciculation and defasciculation of axons within the developing connectives. In addition, glial cells maintain neuronal cell survival by secreting neurotrophic factors (Xiong and Montell, 1995; Booth et al., 2000; Enomoto, 2005).

Based on morphology and position, *Drosophila* CNS glial cells can be placed into three major categories: the surface glia, the neuropile glia and the cortex or cell body glia (Ito et al., 1995; Campos-Ortega and Hartenstein, 1997; Edenfeld et al., 2005). The surface glia represent cells that are closely associated with the CNS surface and these cells possess a pancake-like flat shape. This category is further subdivided in two subtypes: the subperineurial glia that lie beneath the outer surface of the VNC, and the channel glia that lie along the dorsoventral channels. The neuropile glia surround the neuronal fascicles and include the glial cells that associate with the axonal structures: the nerve roots and the neuropile that includes the connectives and commissures. Three subtypes are found in this category: the nerve root glia that are further subdivided into the intersegmental nerve root glia and the segmental nerve root glia, the interface glia and the midline glia. The cell body glia are characterized by a stellate morphology and are located among the neuronal cell bodies in the cortex.

Based on the molecular mode of cell fate specification, *Drosophila* CNS glia can be subdivided into only two classes: the midline glial cells, which require Egfr signaling for development, and the lateral glial cells, which require the activity of the transcription factor *glial cells missing* (*gcm*, also called *glial cell deficient*, *glide*) (Klambt, 2001; Edenfeld et al., 2005). The development of the midline glial cells depends on segment polarity genes that determine positional values and on the gene *single minded* (Crews et al., 1988; Hummel et al.,

1999a,b). The combined action of these factors results in the specific expression of the *Drosophila* Egfr in the midline glial cell progenitors. Binding of Spitz, the *Drosophila* TGF-α homolog, then leads to activation of the well conserved Ras-signaling cascade that influences the balance of two antagonizing transcription factors of the EST family, PointedP2 and Yan. Whereas Yan acts as a negative regulator, the activity of PointedP2 promotes glial differentiation in the midline (Rebay and Rubin, 1995; Scholz et al., 1997).

2.2.2. Intrinsic regulation of glial cell fate: the *gcm* gene

In *Drosophila*, *gcm* encodes a transcription factor that controls the determination of glial versus neuronal fate in cells derived from the neuroectoderm. In *gcm* mutants, cells that normally develop into glia enter a neuronal differentiation pathway leading to a loss of glia and a gain of neurons. In contrast, ectopic expression of GCM throughout the neuroectoderm leads to a profound increase of glial-like cells and has a severe effect on neuronal differentiation: the number of cells that express the neuron-specific marker ELAV is reduced to 5-15% of that in wild type embryos (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Thus, within the nervous system, *gcm* acts as a binary genetic switch, with GCM-positive cells becoming glia and GCM-negative cells becoming neurons. Interestingly, the gliogenic capabilities of *gcm* do not appear to be restricted to the neuroectoderm, since expression in the early mesodermal anlage is also capable to induce at least some glial cell differentiation (Akiyama-Oda et al., 1998; Bernardoni et al., 1998). In vertebrate, two *gcm* homologs, *Gcm1* and *Gcm2* have been identified (Akiyama et al., 1996; Altshuller et al., 1996; Kim et al., 1998; Kammerer et al., 1999; Kanemura et al., 1999). Although they are not expressed at high levels in glial lineages, at least for one of them, *Gcm1*, the potential to induce glial cell differentiation appears to be conserved (Iwasaki et al., 2003). However, glia are not the only cells that express *gcm* during development. In *Drosophila*, *gcm*, together with its closely related homolog *gcm2*, is also required for the differentiation of the plasmatocyte/macrophage lineage of blood cells, or hemocytes (Bernardoni et al., 1997; Lebestky et al., 2000; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). *gcm2* is closely linked to *gcm* on the chromosome, but it has redundant functions with *gcm* and has a minor role in promoting glial cell differentiation.

gcm encodes a nuclear protein that binds to a conserved DNA sequence motive AT(G/A)CGGG(T/C), and it acts as a transcriptional activator (Akiyama et al., 1996; Schreiber et al., 1997, 1998). GCM expression is transient and can be detected as soon as glial

precursors have formed and declines once glial differentiation has started. The control of the initial *gcm* expression is presently not well understood. However, Notch signaling has been shown to influence *gcm* transcription and glial cell differentiation in binary cell fate decisions, with a context-dependent rule that it promotes gliogenesis in the case of neuronal/glial sibling pairs, but has the opposite effect on secondary precursor/sibling pairs (Udolph et al., 2001; Van De Bor and Giangrande, 2001; Umesono et al., 2002). Moreover, it has been shown that *gcm* is able to activate its own transcription through its 5 upstream GCM binding sites (Miller et al., 1998). This positive autoregulation is required for maintenance but not initiation of *gcm* transcription. Recently, two more studies have dissected the cis-regulatory structure of the *gcm* locus (Rogone et al., 2003; Jones et al., 2004). These results indicate that the glial-promoting *cis*-regulatory activity could be divided into at least three components: a general neural component, a lineage-specific component and an autoregulatory component. Hence, *gcm* expression in the CNS requires both lineage-specific activation and general neuronal repression.

2.2.3. Transcriptional control of glial differentiation: downstream of *gcm*

GCM is thought to initiate gliogenesis through the transcriptional activation of glial-specific target genes. However, the whole regulatory network is still poorly understood (Jones, 2005; Fig.2A). Variations of the GCM-binding site (GBS) are found repeated in the putative regulatory regions of a number of glial-specific genes that are dependent on *gcm* expression (Akiyama et al., 1996; Schreiber et al., 1997; Granderath et al., 2000; Freeman et al., 2003). These potential target genes include the glial-specific transcription factors encoded by *reversed polarity* (*repo*) and *pointed* (*pnt*). *repo* expression is *gcm*-dependent and found exclusively in glial cells. Transient expression of *gcm* is followed by maintained expression of *repo*. In *repo* mutant embryos, the migration, survival and terminal differentiation of glial cells are abnormal although the initial glial determination is not affected (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). Thus, *repo* appears to control important aspects of terminal glial differentiation. GCM also induces the expression of PntP1 (an isoform of *pnt*) and Ttk p69 (an isoform of *tramtrack*, *ttk*). Like *repo*, mutations in these genes do not prevent the initiation of glial cell development, but have terminal differentiation defects. *pntP1* promotes different aspects of glial cell differentiation, and is required for the expression of several glial markers (Klaes et al., 1994). In contrast, *ttk p69* acts to repress neuronal differentiation. In *ttk* mutants, glial cells ectopically express neuronal antigens (Giesen et al., 1997). In addition, Ttk p69 inhibits the expression of the pan-neural bHLH

genes *asense* and *deadpan*, which promote the neuronal potential of neural progenitors (Badenhorst, 2001). These data support a model whereby *gcm* promotes glial cell characteristics by initiating the glial-specific transcriptional activators *repo* and *pnt*, while simultaneously repressing neuronal characteristics by activating the transcriptional repressor *ttk*. A recent study further suggests that *repo* may also cooperate with *ttk* to suppress neuronal fates, thereby reinforcing the glial cell fate choice (Yuasa et al., 2003).

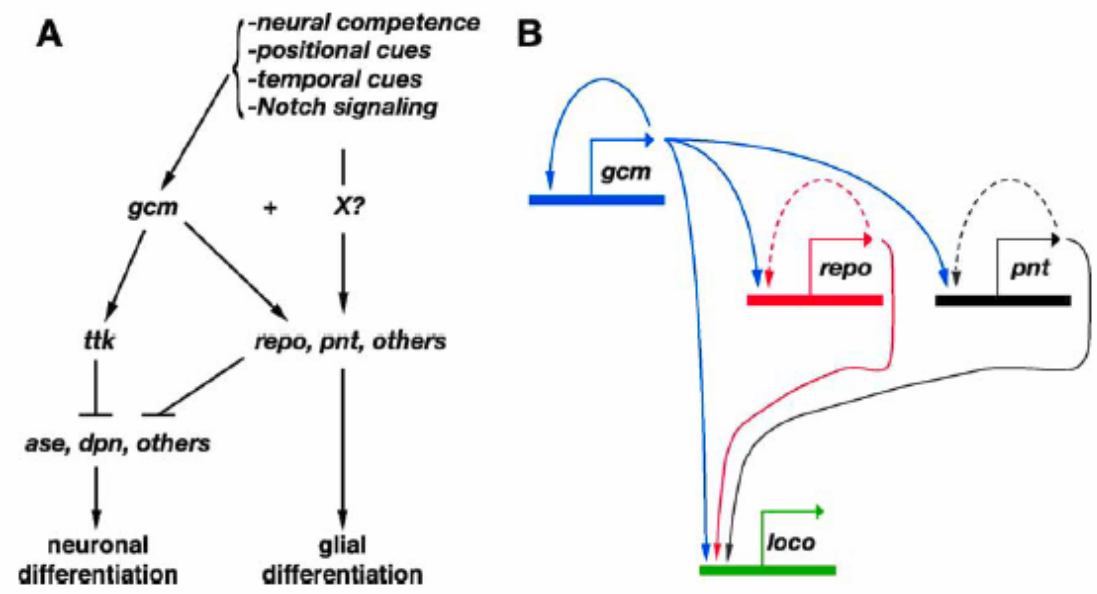

Fig.2. Transcriptional regulatory networks controlling gliogenesis in *Drosophila*. (A) Summary of *gcm* pathway. *gcm* transcription is regulated by multiple inputs in different neural lineages. *gcm* initiates glial cell development by the simultaneous activation of glial differentiation and repression of neuronal differentiation. Additional neural factors (*X*) may be required to activate glial fate. Glial differentiation is promoted by the factors *repo*, *pointed* (*pnt*), and others. Neuronal differentiation is blocked by *tramtrack* (*ttk*) through the repression of neural factors such as *asense* (*ase*) and *deadpan* (*dpn*). *repo* may be required as a co-factor for neuronal repression. (B) Circuit diagram for the transcriptional regulation of the glial-specific gene *loco*. *gcm* cooperates with downstream factors *repo* and *pnt* to initiate and maintain *loco* expression. *gcm* autoregulates to boost its own expression. Dashed lines represent hypothetical autofeedback loops regulating *repo* and *pnt*. Transient expression of *gcm* activates the circuit; *loco* expression is maintained by *repo* and *pnt* (adapted from Jones, 2005).

The expression of *locomotion defects* (*loco*) is an example that *gcm* and its downstream regulators *repo*, *pnt*, and *ttk* appear to act cooperatively at the *cis*-regulatory level to initiate and maintain the expression of glial-specific genes (Fig.2B). The *loco* gene encodes a family member of the regulators of G-Protein signaling proteins expressed in lateral glia (Granderath et al., 1999; Yuasa et al., 2003). A 1.9-kb *cis*-regulatory DNA element of *loco* can direct glial-specific expression of a reporter gene in vivo (Granderath et al., 2000). Scattered in the DNA sequence of this element are three GBSs and an ETS binding site (the consensus site for PntP1 protein). Specific mutation of GBSs causes a complete loss of expression, and mutation of the ETS binding site causes a premature decay of reporter expression. Additionally, ectopic

expression of either *gcm* or *pntP1* drives weak expression of the *loco* reporter, but co-expression of *gcm* and *pntP1* induces robust *loco* reporter expression. These findings suggest that *gcm* is required for the initiation and *pntP1* is required for the maintenance of *loco* expression. Similar studies have shown that *repo* and *pntP1* also cooperate to regulate *loco* expression (Yuasa et al., 2003). Like *pntP1*, *repo* is required for the maintenance of *loco* expression, and ectopic expression of *repo* and *pntP1* together induces stronger ectopic expression of *loco* than either gene can alone. Hence, at least for one gene, a picture is emerging in which GCM initiates the expression of glialspecific genes (along with unknown co-factors), and simultaneously activates downstream transcription factors that cooperate on the same promoters with GCM to activate expression. As GCM is expressed transiently, glialspecific expression is maintained by its downstream transcription factors after GCM disappears. While it remains to be seen if this notion is valid, it would not be surprising if many glial-specific genes are regulated similarly (Jones, 2005).

Considering that only a few downstream targets of *gcm* are known, efforts have been made to identify GCM target genes by taking the advantage of the availability of whole-genome sequences and the development of microarray technology (Egger et al., 2002 in this thesis; Freeman et al, 2003). In turn, the identification of a large number of GCM-regulated genes provides the opportunity to explore the transcriptional regulation of glial cell differentiation at the genomic level.

2.3. The *Drosophila* visual system: a model for studies of axon guidance

There are two distinct phases of neurogenesis, embryonic and postembryonic, during the development of *Drosophila* nervous system. Although a great deal of information has been obtained from intensive studies on the embryonic neurogenesis (see above), less is known about the development of the larval, pupal and adult nervous system. One reason for this could be that genes involved in the embryogenesis or early CNS development are also required for many other developmental processes in diverse tissues. Thus, mutations in these genes are likely to cause pleiotropic developmental defects and/or lethality that preclude the identification of their roles in later nervous system development. Nevertheless, many powerful genetic tools such as mosaic systems have been developed in *Drosophila* and rapid progress has been made in understanding stage-specific gene function in the more complicated postembryonic nervous system (Xu and Rubin, 1993; Lee and Luo, 1999). By

making use of these advantages, in particular, genetic manipulations available for eye-specific mosaic analysis (Stowers and Schwarz, 1999; Newsome et al., 2000), the *Drosophila* visual system (Fig.3) has turned out to be an excellent model system for the study of cellular and molecular mechanisms of axon guidance (Clandinin and Zipursky, 2002; Araujo and Tear, 2003; Tayler and Garrity, 2003; Chotard and Iris, 2004).

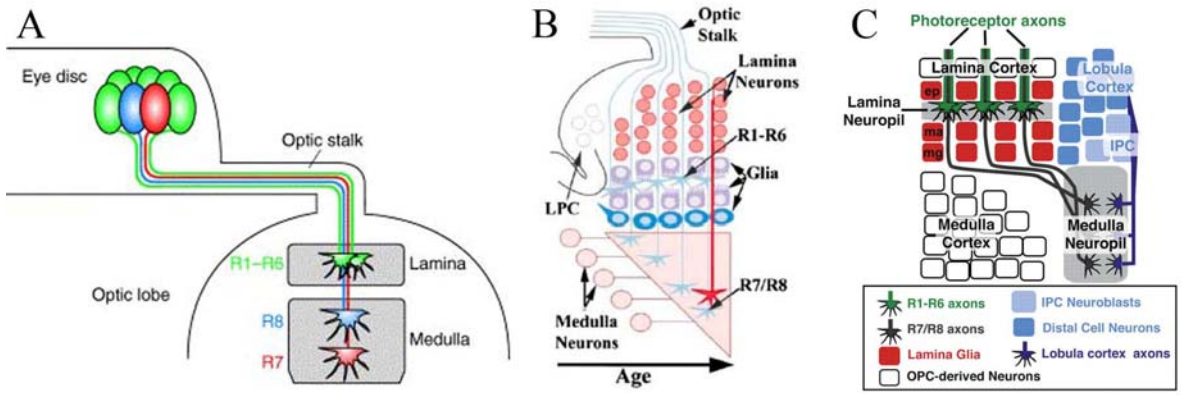

Fig.3. The development of R-cell connections in the *Drosophila* visual system. (A) A single ommatidium containing eight R-cell neurons is shown. The *Drosophila* adult eye contains about 750 ommatidia. R-cell axons project through the optic stalk into the optic lobe, where they contact targets in two ganglia: the lamina and the medulla. The R1–R6 axons (green) stop at their target layer in the lamina, whereas the R7 axon (red) and the R8 axon (blue) continue into the underlying medulla, where they stop in two distinct layers. (B) Horizontal view of third instar visual system (anterior to left). R-cells innervate the lamina and medulla in a sequential fashion. The retina (not shown) develops as a wave sweeping across the retinal primordium. As a consequence, R-cells project into the optic stalk and innervate the lamina and medulla in a sequential fashion. R1-R6 axons in the left part of the lamina are the youngest, with a gradient of increasing developmental age from left to right. Similarly, R7/R8 axons in the medulla are of different ages. Oldest axons are at the bottom of the medulla. As R-cells enter the developing lamina, they come into close contact with the lamina precursor cells (LPC). Signals from R-cell axons induce lamina neuronal development, thereby precisely matching the number of R-cell axon bundles and lamina targets. (C) Horizontal view of third instar visual system (anterior to left). R1-R6 axons (green) terminate between two rows of lamina glial cells (red) in the lamina. Distal cell neurons (blue) generated from the inner proliferation center (IPC) form the anterior edge of the lobula cortex and are located immediately adjacent to the posterior face of the lamina. The maintenance of the lamina/lobula cortex boundary is required for the correct targeting of R1-R6 axons (adapted from Clandinin and Zipursky, 2002; Tayler and Garrity, 2003; Tayler et al., 2004).

2.3.1. Neuronal connectivity in the *Drosophila* visual system

The *Drosophila* visual system comprises the compound eye and the optic ganglia, the lamina, medulla, and the lobula complex. Each region contains numerous different neuronal cell types with distinct morphologies and patterns of connectivity (Fischbach and Dittrich; 1989; Bausenwein et al., 1992; Meinertzhagen and Hanson, 1993). The adult *Drosophila* compound eye is a crystal-like array of roughly 750 light reception units, ommatidia, each containing eight uniquely identifiable photoreceptor neurons, or "R-cells", numbered R1-R8 that project retinotopically to their targets in the optic ganglia (Wolff and Ready, 1993; Morante and Desplan, 2004). These R-cells are arranged in a stereotyped fashion and fall into two classes. R1-R6 cells are outer photoreceptor cells which express a rhodopsin with a broad absorption

in the green range and are involved in detecting motion, while R7 and R8 neurons are inner photoreceptor cells which express rhodopsins that absorb in the ultraviolet and blue range. R7 and R8 neurons are used to see color and detect polarized light (Clandinin and Zipursky, 2002; Araujo and Tear, 2003). The adult eye derives from the eye imaginal disc, a columnar epithelium that begins to differentiate during the third and final stage of larval development. R-cells are specified and assemble into ommatidial clusters in a wave of morphogenesis progressing from posterior to anterior within the disc. Shortly after their formation, every class of R-cells projects axons through the optic stalk to specific synaptic layers in the brain. The R8 growth cone extends first, followed by R1-R6 and then R7. R1-R6 axons terminate in the lamina, forming the lamina plexus, while R7 and R8 axons pass through the lamina and terminate in two separate layers in the medulla (Tayler and Garrity, 2003; Fig.3A).

Target layer selection occurs during larval development. For R1-R6 axons, initially, axons from the same ommatidium extend into the lamina as a single fascicle. Their growth cones terminate in a tight cluster nestled between lamina glia. During pupal development, R1-R6 axons defasciculate and extend laterally to reach six different neighboring targets, lamina cartridges, which are the synaptic units within the lamina (Meinertzhagen and Hanson, 1993; Clandinin and Zipursky, 2002; Morante and Desplan, 2005). As a result, on the one hand, six (R1-R6) axons from a single ommatidium innervate a characteristic pattern of six different targets oriented with respect to the dorsoventral and anteroposterior axes of the lamina target field. On the other hand, the six (R1-R6) cells from six different neighboring ommatidia that receive input from the same point in space project to the same lamina cartridge. In addition, a R7 and R8 pair from yet a different ommatidium also receive the input from this point. For R7 and R8 axons, they also establish layer-specific projections in two separate stages: during the first stage, R8 and R7 axons sequentially target to the R8- and R7-temporary layers respectively; and at the second stage, R8 and R7 growth cones progress synchronously to their destined layers, the M3 and M6 layers in the medulla (Ting et al., 2005). Moreover, lamina monopolar neurons (L1-L5) from a single cartridge project, in turn, to distinct layers within a radially oriented synaptic unit in the medulla, called a column. Each column also contains the synapses of the R7 and R8 neurons. In this way, each medulla column receives input from a single point in space, directly from R7 and R8 and indirectly from R1-R6 via lamina neurons.

The formation of the precise R-cell projection pattern is known to involve complex bidirectional interactions between R-cell axons and different populations of cells in the visual

system. The initial posterior extension of R-cells from the eye disc is dependent upon interactions with a subclass of retinal glial cells, known as retinal basal glia (RBG), which originate in the optic stalk and migrate into the eye disc (Choi and Benzer, 1994). Studies on glial cell migration in the eye suggest that the entry of RBG into the eye disc as well as the timing of this entry is crucial for R-cell axon guidance (Rangarajan et al., 1999; Rangarajan, et al., 2001; Hummel et al., 2002). The signals involved in these interactions are currently unknown, although the secreted proteins Hedgehog and Dpp have been identified as potential candidates. Once R-cell growth cones enter the optic lobe in the brain, they are faced with a choice between two target regions: the lamina and the medulla. The molecular mechanisms that underlie the targeting of R-cell axons have been studied intensively in the photoreceptor neurons of the developing eye, and to a lesser degree in the developing lamina and medulla. Thus, R-cells express a set of genes encoding cell surface receptors, signaling molecules and nuclear factors that have been shown to control target selection in lamina and medulla (Garrity et al., 1996; Garrity et al., 1999; Ruan et al., 1999; Rao et al., 2000; Su et al., 2000; Clandinin et al., 2001; Maurel-Zaffran et al., 2001; Lee et al., 2001; Senti et al., 2000; Kaminker et al., 2002; Lee et al., 2003; Senti et al., 2003; Cafferty et al., 2004; Yang and Kunes, 2004; Prakash et al., 2005; Rawson et al., 2005; Ting et al., 2005).

In the lamina, R-cell axons encounter both neurons and glial cells (Clandinin and Zipursky, 2002; Fig.3B). Lamina neurons L1-L5 are generated from a subpopulation of neuroblasts in the outer proliferation center (Salecker et al., 1998). In a two-step process, neuroblasts give rise to lamina precursor cells (LPCs) and LPCs subsequently complete final divisions to produce mature lamina neurons. During this process, R-cell afferents release signals such as Hedgehog and Spitz to induce lamina neuron development. In turn, LPC progeny assemble into lamina columns which associate with older R-cell axon bundles. However, lamina neurons have been found to be dispensable for the initial targeting of R1-R6 axons. In *hedgehog*^{1} mutant animals, no lamina neurons form but R1-R6 axons are targeted normally (Poeck et al., 2001). In contrast, lamina glial cells, which are generated by glial precursor cells located in two domains at the dorsal and ventral edges of the prospective lamina (Huang and Kunes, 1998), appear to act as intermediate targets for R1-R6 axons and may be an important source of targeting information. When the organization of lamina glia is disrupted, large numbers of R1-R6 axons project through the lamina into the medulla (Poeck et al., 2001; Suh et al. 2002). Recently, it was shown that mature glia migrate into the lamina target field along scaffold axons which serve as migratory guides and the outgrowth of these

scaffold axons is induced by early innervating R-cell axons (Dearborn and Kunes, 2004). In addition to the cellular recognition mechanisms regulating interactions between growth cones and their targets, interactions between afferents also play an important role in contributing to R-cell specificity (Clandinin and Zipursky, 2000; Kaminker et al., 2002).

In this thesis, the *egghead* (*egh*) gene, encoding a glycosyltransferase, is also shown to play a role in the initial targeting of R1-R6 axons (Fan et al. in this thesis). However, unlike what is described above, the requirement of *egh* is restricted to the lobula complex primordium rather than the lamina or medulla.

2.3.2. Compartmental organization in the optic lobe

In the *Drosophila* visual system, the target area of R-cells axons, the optic lobe, comprises the lamina, medulla and lobula complex. Progeny of the outer proliferation center contribute to the lamina and outer medulla, while progeny of the inner proliferation center contribute to the inner medulla and lobula complex. Unlike the lamina and medulla, the mature lobula complex, composed of lobula and lobula plate, does not receive direct input from R-cells in the adult fly brain. However, during optic lobe development, morphogenetic movements of the optic lobe anlagen transiently bring the lobula complex primordium into close apposition to the developing lamina and medulla (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993; Nassif et al., 2003). Given this spatial proximity, correct targeting of R-cell axons and plexus formation in the developing lamina can be influenced by cells of the lobula complex primordium. Recently, the existence of a boundary region between the developing lamina and lobula cortex has been demonstrated, and in the wild type, no intermixing of the two cell populations occurs (Tayler et al., 2004; Fig.3C). This boundary region is the site of molecular interactions between the Slit and Robo family proteins. In the optic lobe, Slit protein is present around lamina glial cells and throughout the medulla neuropile, while Robo proteins are concentrated in the developing lobula cortex. Evidence for a perturbation of the R-cell projection pattern due to invasion of the developing lamina by cells of the lobula cortex has been obtained in *slit* or *robo* loss-of-function mutants, in which the lamina/lobula cortex boundary is disrupted resulting in cell mixing across the two optic lobe compartments (Tayler et al., 2004).

Glial cells are thought to play a major role in the formation and maintenance of many compartments in the central nervous system, and some of the most prominent compartments

in the insect brain, including the optic ganglia, are delimited by sheath-like glial septa (Boyan et al., 1995; Hahnlein and Bicker, 1996; Salecker and Boeckh, 1996; Stollewerk and Klambt, 1997; Younossi-Hartenstein et al., 2003). For the *Drosophila* optic lobe, a classification of glia has been provided for the adult (Eule et al., 1995). Morphogenesis and proliferation of the larval brain glia have also been studied in detail recently (Pereanu et al., 2005). Similar to the central brain, three major classes of glial cells exist in the larval optic lobe. The layer of surface glia covering the central brain continues uninterruptedly over the optic lobe. Cortex glia whose processes wrap around neuronal cell bodies, called satellite glia in the optic lobe, are scattered throughout the emerging cortices of the lamina, medulla and lobula complex. It appears that optic lobe cortex glial processes ensheath individual neuronal precursors from the beginning, rather than forming larger chambers enclosing multiple neurons as in the central brain. Neuropile glia fall into multiple subsets with diverse morphology and function and have been described in other studies (Winberg et al., 1992; Tix et al., 1997). In this thesis, the lamina/lobula cortex boundary is found to be delimited by sheath-like glial cell processes which extend from the lateral surface of the brain to the posterior face of the developing lamina plexus, and the *egh* gene is required for the formation or maintenance of the compartment boundary between lamina glia and lobula cortex. Although the *egh* mutant phenotype is similar to that reported for *slit* or *robo* loss-of-function mutants in the developing optic lobe, our analysis demonstrates that the requirement of *egh* is restricted to the lobula complex primordium (Fan et al. in this thesis).

2.4. Microarrays for genome-wide analysis of gene expression

With the rapid progress in the genome sequencing projects, it has become possible to take advantage of the sequenced genome to decipher biological questions from a global perspective. The *Drosophila* genome is relatively small and is dispersed on four chromosomes: the sex chromosomes (X,Y) and the autosomal chromosomes 2, 3 and 4. The first annotated version of the *Drosophila melanogaster* genome was released in March 2000 (Adams et al., 2000; Myers et al., 2000; Reese et al., 2000). By now, almost the complete euchromatic portion of the genome (~118.4 Mb) has been finished to high quality and the annotation of previous releases was re-evaluated in Release 3 (Celniker et al., 2002) and more recently in Release 4 (http://flybase.bio.indiana.edu/annot/dmel-release4-notes.html). The various genomic projects also promote the development of microarray (GeneChip) technologies. High density microarrays, for the first time, provide biologists with the tool to

investigate simultaneously all the genes from a given genome and allow parallel quantification of their expression levels (Brown and Botstein, 1999). In a high-throughput manner, expression profiling using microarrays appears to be a powerful tool for correlating gene functions with DNA sequences as well (Schena et al., 1995). The use of microarrays for expression profiling is based on two fundamental principles. First, for many genes, a predominant factor underlying changes in expression is an alteration in the abundance of the cognate mRNA (those biological questions involving posttranscriptional regulation are not generally amenable to microarray analysis). Second, only DNA strands possessing complementary sequences can hybridize to each other to form a stable, double-stranded molecule. Microarrays exploit this property through the immobilization of millions of single-strand copies of a gene as individual array elements on a solid support surface. This array surface is then incubated with a mixture of labeled DNA molecules. Only the labeled molecules that represent the same gene as the immobilized DNA elements can form heteroduplexes. By measuring the amount of label at the end of the hybridization, relative transcript abundance levels for each gene can be determined (Deyholos and Galbraith, 2001).

2.4.1 High-density oligonucleotide arrays used in this thesis

Depending on the nature of the probes and how these probes are immobilized, there are two different types of microarrays available for expression profiling. These are spotted DNA microarrays and oligonucleotide arrays, also known as Affymetrix GeneChips. Normally, probes on the spotted arrays are DNA fragments of ~400-2000bp generated by PCR amplification whereas those on the oligonucleotide arrays are oligonucleotide sets with the length of ~25bp representing the genes (Schena et al., 1995). While probes on the spotted arrays are immobilized on the solid surface (membranes or glass) by printing, oligonucleotide probes are synthesized in parallel by using a photolithographic process (Lipshutz et al., 1999; Deyholes and Galbraith, 2001). Thus, oligonucleotide arrays for expression profiling are designed and synthesized based on sequence information alone, without the need for physical intermediates such as clones, PCR products, cDNAs, etc. Using as little as 200 to 300 bases from the coding region or 3' untranslated region, independent 25-mer oligonucleotides are selected (non-overlapping if possible, or minimally overlapping) to serve as sensitive, unique, sequence-specific detectors. According to a set of empirically derived rules, probe design is based on complementarities to the selected gene or EST reference sequence, uniqueness relative to family members and other genes, and an absence of high homology to other RNAs that may be highly abundant in the sample (for example, rRNAs, tRNAs, Alu-like sequences,

housekeeping genes, repetitive sequences). Each gene sequence is represented on the array by a set of 14-20 oligonucleotides (probes) perfectly matching reference sequences. The same set of probes, containing a single nucleotide mismatch in a central position, is also represented on the array. The mismatch probes act as specificity controls that allow the direct subtraction of both background and cross-hybridization signals, and allow discrimination between 'real' signals and those due to non-specific or semi-specific hybridization (Lockhart et al., 1996; Lipshutz et al., 1999). In the first generation of oligonucleotide arrays, all the probes for one specific gene were aligned next to each other whereas, in new oligonucleotide arrays, probes for the same genes are distributed randomly on the array. This is specially designed to control the position effect during hybridization.

Taken together, oligonucleotide arrays have several specific advantages compared to spotted cDNA arrays: (1) They can be designed and made directly from sequence information without physical intermediates; (2) Large numbers of probes are used to increase detection redundancy, meaning there are many 'detectors' per gene so that saturation of hybridization can be avoided; (3) Shorter probes can be targeted to the most unique regions of genes, therefore reducing cross-hybridization so that closely related members of gene families can be discriminated; (4) Involvement of semiconductor techniques and light directed oligonucleotide synthesis allows the construction of arrays with extremely high information content; and (5) Because of how the arrays are manufactured, it is very easy to handle them and the reproducibility of hybridization using the same batch of array is high (Lipshutz et al., 1999). In contrast, the disadvantages of Affymetrix arrays are the high cost and the lack of flexibility inherent in the synthesis process (Deyholos and Galbraith, 2001).

In this thesis, two full genome Affymetrix GeneChips were used for global gene expression profiling in *Drosophila* embryos. (1) The first full genome-GeneChip available was a custom-designed *Drosophila* GeneChip (roDROMEGAa; Affymetrix Inc.). It contains 14,090 sequences representing 13,369 genes from the Release 1.0 of the annotated *Drosophila* genome (Egger et al., 2002 in this thesis). (2) The second full genome array used was the commercial DrosGenome1 (Affymetrix, cat# 900 335). This array is also based on the Release 1.0 of the *Drosophila* genome. Sequences on the array represent more than 13,500 predicted transcripts as well as different control genes (Montalta-He et al. in this thesis). The probes on roDROMEGAa were selected from the coding region of the genes. In contrast, for DrosoGenome1, probes were specifically chosen from the 3' untranslated region for two

reasons: firstly, sequences in the 3' UTR have been shown to be more gene-specific which will theoretically lower the chance of unspecific and cross hybridization; secondly, this complements very well to the 3' bias of target preparation that contributes to the accuracy of the microarray experiments.

2.4.2 Design issues for microarray experiments

There is no doubt that microarrays are powerful and efficient tools to quantify and compare gene expression on a large scale. However, as with all large-scale experiments, microarray experiments can be costly in terms of equipment, consumables and time. Moreover, there are inherent biological factors that influence whether the resulting experiment is to be maximally informative, given the effort and the resources (Yang and Speed, 2002). Therefore, careful attention to experimental design is particularly important to avoid potential biases and improve the efficiency and reliability of the data obtained. Many aspects should be considered during the design of microarray experiment, for example, considerations about the biological question, choice of arrays, replicates used, ways of sampling and data analysis and interpretation. Here I mainly focus two aspects related to this thesis: sample heterogeneity and data validation.

One of the major problems that hinders the further application of microarrays is the relatively low level of validation attained. A main reason accounting for this drawback appears to be the complexity of the tissue when multicellular organisms are used for microarray experiments. Consequently, biologically relevant changes in gene expression level may be very subtle so that small differences may be averaged out in the overall signal and missed. This is especially prominent when studying neural tissue because the intrinsic heterogeneity of the tissue samples used causes a signal-to-noise problem for the specific detection of gene expression in a given microarray experiment (Barlow and Lockhart, 2002; Griffin et al., 2003; Henry et al., 2003). One way to solve the problem of tissue heterogeneity is to purify specific cell types from complex tissue such as a developing nervous system. There have been several successful examples of microarray experiments based on purification of specific cell types. These include the application of Laser Captured Microdissection (LCM), Fluorescent Associated Cell Separation (FACS), mRNA-tagging or single cell transcript profiling (Bryant et al., 1999; Mills et al., 2001; Roy et al., 2002; Luzzi et al., 2003; Tietjen et al., 2003). These all demonstrate that access to a homogeneous population of specific cell types facilitates the application of microarray analysis in developmental biology. In this thesis, we applied the

technique of magnetic cell separation (MACS) to isolate neuroectoderm cells from *Drosophila* embryos for microarray analysis of *gcm* gene action in neurogliogenesis (Montalta-He et al. in this thesis). In vivo validation studies of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed high rates of verification.

Typically, microarray studies generate large and complex multivariate data sets and some of the greatest challenges lie not in generating these data but in the development of computational and statistics tools to analyze the large amounts of data. Moreover, in order to answer a biological question, these data are only of value when validated with independent in vivo follow-up experiments. Currently, several methods have been used for the validation of microarray data. They are Northern blot, Western blot, real time RT-PCR, in situ RNA hybridization and antibody immunostaining. Among these, Northern blot, Western blot and RT-PCR are more quantitative and high-throughput than in situ hybridization and antibody immunostaining. But in situ hybridization and antibody immunostaining not only can confirm the changes qualitatively but also give biological information concerning the spatial and temporal expression pattern of the genes, which might lead directly to the function of the gene and help to exclude experimental artifacts (Barlow and Lockhart, 2002). Given that changes in gene expression measured by microarrays can be spatially ubiquitous or ectopic, it seems that the combination of quantitative methods combined with in situ hybridization or antibody immunostaining leads to much better validation results. In the fly community, efforts have been directed to use high-throughput RNA in situ hybridization to assemble a database of gene-expression patterns for embryonic development of *Drosophila* (Tomancak et al., 2002; Montalta-He and Reichert, 2003). This database will definitely facilitate the validation of microarray data and consequently the application of microarrays in research. In addition, the efficiency of microarray data validation may also somewhat depend on the individual criteria of data selection. These criteria are generally made by the parameter (threshold filter value) settings of the software used for microarray data analysis. The importance of parameters such as the Average Difference value (Avg Diff), the Fold Change levels (FCs) and the p-value are discussed in this thesis.

2.5. This thesis

The generation of cellular diversity and the establishment of cellular specification is a fundamental process during nervous system development. In *Drosophila*, *glial cells missing (gcm)*, encoding a transcription factor, is a key control gene of embryonic gliogenesis. Although GCM is thought to initiate gliogenesis through the transcriptional activation of glial-specific target genes, the whole regulatory network is still poorly understood. To identify *gcm* downstream genes in a comprehensive manner, in the first part of this thesis, genome-wide oligonucleotide arrays were used to analyze differential gene expression in wild type embryos versus embryos in which *gcm* was misexpressed throughout the neuroectoderm. Transcripts were analyzed at two defined temporal windows during embryogenesis. Hundreds of genes that were differentially expressed following *gcm* misexpression were found and thus are potentially involved in aspects of glial development. This first genome-wide analysis of gene expression events downstream of a key developmental transcription factor presents a novel level of insight into the repertoire of genes that initiate and maintain cell fate choices in CNS development (Egger et al., 2002 in this thesis).

Although microarrays are powerful and efficient tools to quantify and compare gene expression on a large scale, a low rate (~30%) of in vivo verification was found during the further analysis of the initial microarray data we obtained for *gcm* downstream genes. One possible reason of this is the complexity of the tissue, the whole embryo, used for the microarray experiments. One way to solve problems of tissue heterogeneity is to reduce as much as possible the irrelevant tissues. To achieve this, in the second part of this thesis, a combination of genetic labeling and magnetic cell sorting was used to isolate neuroectodermal cells from *Drosophila* embryos for microarray analysis of *gcm* gene action in neurogliogenesis. Validation studies of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed a high rate of in vivo verification of more than 80%. Given that the magnetic cell separation technique (MACS) only requires simple experimental settings in comparison to other cell sorting techniques such as Laser Captured Microdissection (LCM), Fluorescent Associated Cell Separation (FACS), single cell transcript profiling and mRNA-tagging, this study should facilitate the application of microarray techniques in *Drosophila* (Montalta-He et al. in this thesis).

One of the putative *gcm* downstream genes that was identified by microarray analysis and validated by in situ hybridization is the *egghead* (*egh*) gene. It encodes a Golgi/ER-localized

glycosyltransferase and is known to play important roles in oogenesis and embryonic epithelial development. In the final part of this thesis, the role of *egh* in *Drosophila* visual system development was investigated. During larval development, the formation of the R-cell (photoreceptor neuron) projection pattern is known to involve complex bidirectional interactions between R-cell axons and different populations of cells in the target area. Although the molecular mechanisms that underlie the targeting of R-cell axons have been studied in the developing lamina, medulla and especially in the photoreceptor neurons of the developing eye, little is known about the possible role of the lobula complex primordium which transiently abuts the lamina and medulla in the developing larval brain. Our findings show that in *egh* loss-of-function mutants, R-cell axons form a disorganized projection pattern characterized by defects in the lamina plexus and aberrant projection of some R1-R6 axons through the lamina and into the medulla. Moreover, in the absence of *egh*, the arrangement of lamina glia and the lamina/lobula cortex boundary are disrupted which correlate spatially with defects in the associated lamina plexus. Notably, this *egh* mutant phenotype is similar to that reported recently for *slit* or *robo* loss-of-function in the developing optic lobe. Further genetic analysis involving mosaics demonstrates that these defects are not due to a loss of *egh* function in the eye or in the neurons and glia of the lamina. Instead, clonal analysis and cell-specific genetic rescue experiments show that *egh* is required in the cells of the lobula complex primordium. Detailed analysis of the compartment boundary region in *egh* mutants suggests that perturbation of glial sheaths occurs at the interface between lamina glia and distal cells of the lobula cortex. Cell mixing across the lamina/lobula cortex boundary occurs, and neurons of the lobula cortex invade the developing lamina at the site of lamina plexus formation disrupting the pattern of lamina glia and resulting in inappropriate R1-R6 axonal projections. This study thus uncovers the *egh* gene is required in the lobula complex primordium for the compartmentalization of *Drosophila* visual centers and underscores the important role of the lamina/lobula cortex boundary in correct targeting of R1-R6 axons (Fan et al. in this thesis).

3. Gliogenesis in *Drosophila*: genome-wide analysis of downstream genes of *glial cells missing* in the embryonic nervous system

Boris Egger[1], Ronny Leemans[1], Thomas Loop[1], Lars Kammermeier[1], Yun Fan[1], Tanja Radimerski[1], Martin C. Strahm[2], Ulrich Certa[3], and Heinrich Reichert[1]

[1]Biozentrum/Pharmazentrum, University of Basel, CH-4056 Basel, Switzerland.
[2]Roche Bioinformatics and [3]Genetics Pharmaceuticals Division, F. Hoffmann-La Roche, Ltd, CH-4070 Basel, Switzerland.

Running Title: *gcm* downstream genes

Key Words: *glial cells missing*, glial cell, nervous system, oligonucleotide microarray, gene expression

Development 129 (2002), 3295-3309.

Summary

In *Drosophila*, the *glial cells missing* (*gcm*) gene encodes a transcription factor that controls the determination of glial versus neuronal fate. In *gcm* mutants, presumptive glial cells are transformed into neurons and, conversely, when *gcm* is ectopically misexpressed, presumptive neurons become glia. Although *gcm* is thought to initiate glial cell development through its action on downstream genes, which execute the glial differentiation program, little is known about the identity of these genes. To identify *gcm* downstream genes in a comprehensive manner, we used genome-wide oligonucleotide arrays to analyze differential gene expression in wild type embryos versus embryos in which *gcm* was misexpressed throughout the neuroectoderm. Transcripts were analyzed at two defined temporal windows during embryogenesis. During a first period of initial *gcm* action on determination of glial cell precursors, over 400 genes were differentially regulated. Among these are numerous genes that encode other transcription factors, underscoring the master regulatory role of *gcm* in gliogenesis. During a second later period when glial cells had already differentiated, over 1200 genes were differentially regulated. Most of these genes, including many genes for chromatin remodeling factors, and cell cycle regulators, were not differentially expressed at the early stage indicating that the genetic control of glial fate determination is largely different from that involved in maintenance of differentiated cells. At both stages, glial-specific genes were upregulated and neuron-specific genes were downregulated supporting a model whereby *gcm* promotes glial development by activating glial genes while simultaneously repressing neuronal genes. Also at both stages, numerous genes that were not previously known to be involved in glial development were differentially regulated and, thus, identified as potential new downstream targets of *gcm*. For a subset of the differentially regulated genes, tissue-specific in vivo expression data were obtained which confirmed the transcript profiling results. This first genome-wide analysis of gene expression events downstream of a key developmental transcription factor presents a novel level of insight into the repertoire of genes that initiate and maintain cell fate choices in CNS development.

Introduction

During CNS development, two major cell types are generated, namely neurons and glial cells. These can be generated either by common precursors (neuroglioblasts) or by precursors that are specialized to produce either neurons (neuroblasts) or glial cells (glioblasts) (Akiyama-Oda et al., 1999; Bernardoni et al., 1999; Miller et al., 1999; Gage, 2000; Malatesta et al., 2000; Qian et al., 2000; Noctor et al., 2002). Neuroglial development has been studied in detail in *Drosophila*, where each embryonic neuromere consists of approximately 60 glial cells and 700 neurons (Klambt and Goodman, 1991; Ito et al., 1995; Schmidt et al., 1997; Jones, 2001). In *Drosophila*, neuroblasts divide asymmetrically to produce ganglion mother cells, which divide once to produce two neurons (Doe and Skeath, 1996), whereas glioblasts produce only glial cells. Glial and neuronal cell lineages in *Drosophila* also derive from neuroglioblasts, which divide asymmetrically to produce a neuroblast and a glioblast.

In *Drosophila*, the *glial cells missing* (*gcm*) gene encodes a transcription factor that controls the determination of glial versus neuronal fate in neuroectodermally derived cells (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Wegner and Riethmacher, 2001). In *gcm* mutants, cells that normally develop into glia enter a neuronal differentiation pathway leading to a loss of glia and a gain of neurons. In contrast, targeted *gcm* misexpression in neural progenitors leads to an increase of glial cells at the expense of neurons. Neither a specific embryonic stage nor a neural "ground state" appear necessary for *gcm* action since misexpression of *gcm* in epidermis or mesoderm suppresses normal cell fate and causes cells to adopt a glial fate (Akiyama-Oda et al., 1998; Bernardoni et al., 1998). Mesectodermal midline glial cells do not require *gcm* function (Granderath and Klambt, 1999).

The molecular mechanisms of *gcm* action in glial development are poorly understood. Clearly, *gcm* transcription factor action depends on its target genes, however, relatively few genes, such as the *reversed polarity* (*repo*) gene, are known to act downstream of *gcm* (Akiyama et al., 1996). To identify *gcm* downstream genes in a comprehensive manner, we carried out a novel functional genomic approach using genome-wide oligonucleotide arrays. These arrays were used to analyze the transcripts in wild type embryos versus embryos in which *gcm* was misexpressed throughout the CNS. Tissue-specific misexpression was achieved by using a *scabrous-GAL4* (*sca-GAL4*) line (Klaes et al., 1994) to drive *gcm*

expression throughout the embryonic neuroectoderm. Transcripts were analyzed at two defined temporal windows during embryogenesis. First, during a period of initial *gcm* action on determination of glial cell precursors and second, during a later period when glial cells have already differentiated. In both cases, we find significant changes in transcript abundance for hundreds of identified genes following *gcm* misexpression. Remarkably, over half of these genes has not yet been studied in any in vivo context in *Drosophila*. All of these identified genes are potential direct or indirect downstream targets of *gcm* and may, thus, be involved in regulating glial cell fate.

Materials and methods

Flies

The wild type was Oregon-R. For targeted misexpression of *gcm*, virgin females from *scabrous-GAL4* (Klaes et al., 1994) were crossed to *yw*; *UAS-gcm*; *UAS-gcm* males (Jones et al., 1995). For *gcm* loss-of-function studies the null allele *gcmΔP1* (Jones et al., 1995) was used balanced over *CyO-wglacZ*. Homozygous mutants were identified by absence of either anti-RK2/REPO or anti-βGAL staining. Stocks were kept on standard medium at 25°C. After a 1 hr pre-collection, wild type and *sca-gcm* embryos were collected in parallel for 1 hr and staged to 6-7 hrs AEL (stage 11) or to 13-14 hrs AEL (late stage 15/early stage 16). Stages are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

Arrays and hybridization

A custom-designed *Drosophila* oligonucleotide array (roDROMEGAa, Affymetrix, Santa Clara, CA) was used (Montalta-He et al., 2002). It contains 14,090 sequences representing 13,369 single transcripts encoding *Drosophila* proteins deposited in SWISS-PROT/TrEMBL databases (Celera Genome/BDGP Release no. 1) (Adams et al., 2000) as well as prokaryotic and custom chosen control sequences. Each sequence is represented on the array by a set of 14 oligonucleotide probes of matching sequence and 14 probes with a single nucleotide mismatch. The Average Difference (Avg Diff) between the perfect match hybridization signal and the mismatch signal is proportional to the abundance of a given transcript (Lipshutz et al., 1999). RNA was isolated, labeled and hybridized to the array as described in (Leemans et al., 2001). Four replicates were performed for each experimental condition.

Data analysis

Data acquisition and processing was as described in (Leemans et al., 2001). For quantification of relative transcript abundance, the Average Difference value (Avg Diff) was used. All arrays were normalized against the mean of the total sums of Avg Diff values across all 16 arrays. For differential transcript imaging, only transcripts that had significant changes in Avg Diff ($p \leq 0.01$; unpaired t-test) in the 1.5-fold and above range were considered, and then only if the mean Avg Diff for the transcript was ≥ 50 in at least one condition.

In situ hybridization and immunocytochemistry

In situ hybridization was according to (Tautz and Pfeifle, 1989). Embryos were mounted in Canada balsam (Serva) and photographed with a Prog/Res/3008 digital camera (Kontron, Zürich) on a Zeiss Axioskop microscope with differential interference contrast optics. Immunocytochemical experiments were done according to (Therianos et al., 1995; Leemans et al., 2001). The primary antibodies were rat anti-RK2/REPO 1:1000 (Campbell et al., 1994), mouse anti-TEN-M 1:250 (Baumgartner et al., 1994), rabbit anti-EY 1:500 (Kammermeier et al., 2001), mouse anti-WRAPPER 1:5 (Noordermeer et al., 1998) and goat anti-HRP (FITC-conjugated) 1:20 (Jackson Immunoresearch). For fluorescent labelings secondary antibodies were Alexa568 and Alexa488 conjugated, all 1:150 (Molecular Probes). For laser confocal microscopy, a Leica TCS SP was used.

Results

Targeted misexpression of *gcm* in the embryonic neuroectoderm results in a switch from neuronal to glial fate

For targeted misexpression of *gcm* in the neuroectoderm of *Drosophila* embryos, a *scaGAL4* enhancer trap line (Klaes et al., 1994) was crossed with an *UAS-gcm* responder line (*sca-gcm*) (Jones et al., 1995). This resulted in ectopic *gcm* expression in the embryonic CNS starting from embryonic stage 9 and diminishing, similar to endogenous *gcm* expression, at embryonic stage 15. Although misexpression of *gcm* starts at stage 9 in *sca-gcm* embryos, ectopic expression of the *repo* gene, a known direct target of *gcm,* was not seen before stage 11, similar to endogenous *repo* expression.

In order to identify genes that are either direct *gcm* target genes or among the initial set of downstream genes of *gcm*, we carried out a first genome-wide analysis of differential gene expression at embryonic stage 11 when the first glial marker, the direct *gcm* target gene *repo,* is expressed. In the wild type during stages 10-11, two small groups of neuroectodermal cells per hemisegment transiently express *gcm*, and a single *gcm*-expressing glial precursor delaminates from each of these groups and expresses the *repo* gene (Fig. 1A,C) (Hosoya et al., 1995; Jones et al., 1995). In contrast, in *sca-gcm* embryos during stages 10-11, all of the cells in the neuroectoderm express *gcm* (Fig. 1B) and, in consequence, most of the neural precursor cells become REPO positive (Fig. 1D). With the exception of altered gene expression in cells of the neuroectoderm, neither gene expression changes outside of the neural lineage nor any obvious morphological changes are seen in these stage 11 *sca-gcm* embryos.

In order to identify also additional indirect downstream genes of *gcm* that act further along in the genetic cascade of *gcm* action, we carried out a second genome-wide transcriptional analysis at embryonic stage 15/16 when glial cells are differentiated. In the wild type at stage 15/16, approximately 700 neurons and 60 glial cells per neuromere have differentiated, and the glial cells (with the exception of midline glia) are REPO positive (Fig. 1E) (Ito et al., 1995). In contrast, in stage 15/16 sca-*gcm* embryos, 80%-90% of the cells in the CNS express REPO protein (Fig. 1F) and have a glial morphology (Hosoya et al., 1995). Correspondingly,

the number of cells expressing the neuronal marker *embryonic lethal*, *abnormal vision* (*elav*) in these *sca-gcm* embryos is reduced by approximately 90% (data not shown) (Hosoya et al., 1995), and a striking reduction of the CNS axon scaffold is observed. In addition to the pronounced changes in the number of glial versus neuronal cells, stage 15/16 *sca-gcm* embryos also show defects in ventral nerve cord condensation and in peripheral innervation. No other gross morphological changes were seen in these stage 15/16 *sca-gcm* embryos.

Overview of differential gene expression following *gcm* misexpression

Analysis of differential gene expression in stage 11 and stage 15/16 *sca-gcm* versus wild type embryos was carried out with oligonucleotide arrays representing 13,369 annotated *Drosophila* genes. This corresponds to virtually all of the currently annotated genes of the *Drosophila* genome sequence (Adams et al., 2000). For each embryonic stage, 2 x 4 replicate oligonucleotide arrays were used to detect transcript levels in *sca-gcm* embryos as compared to wild type controls. Only transcripts, which show an expression level fold change (FC) ≥ 1.5 or ≤ –1.5 at significance values of p≤ 0.01 (t-test) were considered as differentially expressed (see Materials and methods). A complete list of all of these genes, as well as their quantitative fold change values is given at www.ncbi.nlm.nih.gov/geo/.

At stage 11, we detected 417 transcripts with differential expression values in *sca-gcm* embryos as compared to wild type. This corresponds to ~3% of the transcripts on the array. Approximately the same number of transcripts have increased (n=219) and decreased (n=198) abundance levels, indicating that *gcm* causes both activation and repression of downstream gene transcription. At stage 15/16, we detected 1259 genes with differential expression values in *sca-gcm* embryos compared to wild type. This corresponds to ~9% of the transcripts on the array. Thus, markedly more transcripts are differentially expressed at stage 15/16 than at stage 11. Again, approximately the same number of transcripts are upregulated (n=609) and downregulated (n=650).

For an overview, all differentially expressed genes of known or predicted molecular function were grouped into functional classes. At stage 11, 199 transcripts of known function belonging to 13 functional classes are differentially expressed in *sca-gcm* embryos (Table 1). The two functional classes with the largest number of differentially regulated transcripts are enzymes (78) and nucleic acid binding proteins (44) including 26 transcription factors. At

stage 15/16, 614 transcripts of known function belonging to 15 functional classes are differentially expressed in *sca-gcm* embryos (Table 1). The two functional classes with the largest number of differentially regulated transcripts are again enzymes (249) and nucleic acid binding proteins (96) including 38 transcription factors. Strikingly, however, at both stages, the majority of the differentially expressed transcripts are of (currently) unknown function; 218 (52%) at stage 11 and 645 (51%) at stage 15/16.

Differential expression of genes encoding transcription factors

The fact that *gcm* acts as a fate switch and key regulator of gliogenesis suggests that *gcm* might control a number of other transcription factors, which in turn would regulate the expression of their own downstream genes. Transcript profiling of *gcm* misexpression indicated that *gcm* does indeed control the expression of numerous other transcription factors. These transcription factor encoding genes and a quantification of their changes in expression level are shown in figure 2.

In stage 11 embryos, 26 genes encoding transcription factors are differentially regulated by targeted *gcm* misexpression (11 upregulated, 15 downregulated). The *gcm* gene has the highest expression level increase (8.6 fold), in accordance with our experimental procedure. (The *gcm* gene also has a high absolute level of expression at this stage; see color coding in figure 2.) The *repo* gene, a known direct target of *gcm* (Akiyama et al., 1996), has the second highest increase in expression level (4.8-fold). Many of the other upregulated transcription factor genes such as *zinc finger homeobox-2* (*zhf-2*), *u-shaped* (*ush*) and the Enhancer of Split complex-member *HLHm3* are known to act in different aspects of embryonic nervous system development (Delidakis and Artavanis-Tsakonas, 1992; Lundell and Hirsh, 1992; Cubadda et al., 1997). Genes of the Enhancer of split complex, for example, act during neural versus epidermal cell fate decision (Jennings et al., 1994), and in the mouse, Enhancer of split members *Hes1* and *Hes5* have been shown to enhance glial cell fate (Furukawa et al., 2000; Hojo et al., 2000). Among the transcription factors with decreased expression levels are *engrailed* (*en*) and *ventral veins lacking/drifter* (*vvl/drf*), which are expressed in a subset of neuronal precursor cells and are also involved in midline glial cell development, but not in lateral glial cell development (Condron et al., 1994; Anderson et al., 1995). Other genes encoding transcription factors with decreased expression levels are *sloppy paired 1* (*slp1*), *goosecoid* (*gsc*), and *forkhead domain 96Cb* (*fd96Cb)*, which are expressed in subsets of

neural precursor cells (Hacker et al., 1992; Hahn and Jackle, 1996; Bhat et al., 2000). Moreover, the *scratch* (*scrt*) transcription factor, a pan-neuronal gene encoding a zinc finger protein that promotes neuronal development and can induce additional neurons when ectopically expressed (Roark et al., 1995), also shows decreased expression levels.

In stage 15/16 embryos, 38 genes encoding transcription factors are differentially regulated by targeted *gcm* misexpression (18 upregulated, 20 downregulated). As expected, *gcm* has the highest expression level increase (18.2 fold). (The absolute level of expression of the *gcm* gene is now an order of magnitude lower at this stage than at stage 11; see color coding in figure 2.) In contrast to high REPO protein levels in stage 15/16 *sca-gcm* embryos (Fig 1F), significant expression of *repo* transcripts is not detected at this stage. Several genes encoding transcription factors, which are expressed in specific neurons, such as *eyeless* (*ey*) and *Ultrabithorax* (*Ubx*) (Hirth et al., 1998; Kammermeier et al., 2001), are downregulated. Moreover, several members of the Enhancer of split complex such as *HLHmbeta*, *HLHm7*, and *E(spl)*, are downregulated at stage 15/16, in contrast to stage 11; in addition to a role in early neurogenesis, these genes continue to be expressed in the normal developing nervous system of the wild type at later embryonic stages (Wech et al., 1999).

The marked increase in the number of affected transcripts at stage15/16 is due in part to the fact that numerous genes encoding transcription factors belonging to the basal transcription machinery are differentially regulated at this stage. Among these are *TfIIFbeta*, *Taf55*, *TfIIB*, *Taf60*, *Taf80*, and *Taf150* (Frank et al., 1995; Lee et al., 1997; Aoyagi and Wassarman, 2000). Moreover, among the upregulated genes encoding transcription factors several are involved in chromatin remodeling such as the brahma complex or associated genes, *dalao* (*dalao*), *Brahma associated protein 60 kp* (*Bap60*), *Snf5-related 1*(*Snr1*) and *absent, small or homeotic disc 2* (*ash2*) (Francis and Kingston, 2001). This suggests that the maintenance of glial cell differentiation at later embryonic stages involves chromatin remodeling as well as the regulation of global transcriptional processes.

In addition to the above mentioned genes for transcription factors involved in chromatin remodeling, a number of genes encoding other proteins which bind to DNA/chromatin are influenced by *gcm* misexpression. These genes and a quantification of their expression level changes are shown in figure 4A. In stage 11 embryos, 7 genes encoding chromatin binding proteins are differentially regulated (3 upregulated, 4 downregulated), and at stage 15/16

embryos, 26 genes encoding chromatin binding proteins are differentially regulated (17 upregulated, 9 downregulated). Prominent among the upregulated genes thought to be involved in chromatin condensation and segregation are *gluon* (*glu*) (Steffensen et al., 2001) and the two DNA replication factor genes *Mini chromosome maintenance 6* and *7* (*Mcm6*) and (*Mcm7*) (Ohno et al., 1998). Among the genes with downregulated expression are the three Sox-related genes *sox-like* (*sox-like*), *Sox box protein 14 (Sox 14)* and *Dichaete* (*D*) which encode DNA bending proteins. *D* is known to be expressed in neural precursor cells and in midline glial cells (Soriano and Russell, 1998; Sanchez-Soriano and Russell, 2000).

Only 4 genes encoding DNA binding proteins, including 2 that encode transcription factors, are differentially expressed in both early and late stage *sca-gcm* embryos. This represents only 4% of the genes for DNA binding proteins that are differentially expressed in these embryos. This finding, which in qualitative terms holds for all other functional classes of differentially expressed genes, indicates that the molecular genetic mechanisms of early glial fate determination are largely different from those involved in the later maintenance of differentiated glial cells.

Differential expression of genes encoding kinases and phosphatases

Cell-cell interactions between neuronal and glial cells are crucial for key cellular processes such as metabolic exchange, extrinsic signaling and electrical insulation. The switch from neuronal to glial fate caused by *gcm* misexpression is, therefore, likely to affect genes that encode proteins involved in cell-cell signaling. Transcript imaging analysis of *gcm* misexpression indicates that *gcm* does indeed control numerous genes that encode kinases and phosphatases involved in signaling pathways. A list of these genes as well as a quantitative representation of their changes in expression levels is shown in figure 3. Once again, a marked increase in the number of affected transcripts was observed at stage 15/16 as compared to stage 11.

In stage 11 embryos, 13 genes encoding kinases or phosphatases are differentially regulated by *gcm* misexpression (8 upregulated, 5 downregulated). Among the genes with increases in transcript abundance is *heartless* (*htl*) which encodes a fibroblast growth factor (FGF) receptor expressed in lateral glial cells (Shishido et al., 1997). Conversely the *Epidermal growth factor receptor* (*Egfr*) shows a decrease in transcript abundance; the *Egfr* pathway is

implicated in midline glial cell development (Scholz et al., 1997). Decreased expression is also observed for *shaggy* (*sgg*), which encodes a protein kinase, and for *skittles* (*sktl*), which encodes a putative phosphatidylinositol-4-phosphate 5-kinase. Cells in *sgg* mutant embryos can not adopt early epidermal fates and instead develop characteristics of CNS cells (Bourouis et al., 1989). Mutations in *sktl* cause abnormal development in the PNS (Prokopenko et al., 2000).

In stage 15/16 embryos, 59 genes encoding kinases or phosphatases are differentially regulated by *gcm* misexpression (29 upregulated, 30 downregulated). A number of genes involved in cell proliferation and mitotic division are upregulated. These included *polo* (*polo*), *discs overgrown* (*dco*), *smallminded* (*smid*) and *Nek2* (*Nek2*) (Llamazares et al., 1991; Schultz et al., 1994; Long et al., 1998; Zilian et al., 1999). In contrast, genes involved in aspects of neuronal development such as axogenesis and synaptogenesis are downregulated. Among these are *derailed* (*drl*), *Neuron-specific kinase* (*Nrk*), and *Cdk5 activator-like protein* (*Cdk5alpha*). The *drl* gene is involved in axonal guidance including routing across the midline (Bonkowsky et al., 1999). *Nrk* is specifically expressed in the embryonic CNS (Oishi et al., 1997). *Cdk5alpha* controls multiple aspects of axon patterning (Connell-Crowley et al., 2000). The only gene in this class that is known to be involved in glial differentiation is *htl*, which is upregulated at stage 11, and remains upregulated in stage 15/16 embryos albeit at a lower level.

Differential expression of genes involved in cell cycle regulation

As mentioned above, a number of chromatin binding protein and kinase/phosphatase encoding genes involved in cellular proliferation and in mitotic division are upregulated by *gcm* misexpression. This suggests that other genes involved in proliferation and division may also be affected by *gcm* misexpression. Transcript profiling of *gcm* misexpression indicates that *gcm* does indeed influence genes that encode cell cycle regulators. These genes and a quantitative representation of their changes in expression levels are shown in figure 4B.

10 genes encoding cell cycle regulators are differentially regulated by *gcm* misexpression (7 upregulated, 3 downregulated) in stage 15/16 embryos. For example, increases in transcript abundance are found for *Cyclin B* (*CycB*), *Cyclin A* (*CycA*), and *Cyclin D* (*CycD*). These genes encode regulators of cyclin dependent kinases that act in different phases during mitotic

cell cycles (Follette and O'Farrell, 1997). In contrast, and rather unexpectedly, a marked decrease in transcript abundance is found for *Cyclin E* (*CycE*). *CycE* is essential for S-phase progression and its downregulation leads to the arrest of cell proliferation (Knoblich et al., 1994). Remarkably, in the earlier embryonic stage 11, none of the genes in the class of cell cycle regulators are influenced by *gcm* misexpression.

Differential expression of genes encoding cell adhesion molecules

Several cases for *gcm*-dependent regulation of genes encoding cell adhesion molecules were observed. These genes as well as a quantitative representation of their expression level changes are shown in figure 4C. At stage 11, 4 genes encoding cell adhesion molecules are differentially regulated by *gcm* misexpression (2 upregulated, 2 downregulated). At stage 15/16, 19 genes encoding cell adhesion molecules are differentially regulated by *gcm* misexpression (4 upregulated, 15 downregulated).

A striking example for a gene with a marked increased transcript level (13.6 fold) in stage 15/16 embryos is *wrapper*. *wrapper* encodes a cell adhesion molecule that is expressed in midline glial cells and in late stages also in lateral glial cells (Noordermeer et al., 1998). Genes with decreased transcript levels in stage 15/16 embryos that are mainly expressed in neurons are *Tenascin major* (*Ten-m*), *Cadherin-N* (*CadN*) and *neuromusculin* (*nrm*). All three act during axogenesis and synaptogenesis (Kania et al., 1993; Baumgartner et al., 1994; Levine et al., 1994; Iwai et al., 1997). The fact that most of the affected genes in the cell adhesion class show *gcm*-dependent decreased transcript levels could reflect the large diversity of cell adhesion molecules expressed by neurons.

***gcm* misexpression may influence genes that act in the hemocyte lineage**

In addition to its key role in gliogenesis, *gcm* also functions in a mesodermal lineage that gives rise to hematopoietic cells (Bernardoni et al., 1997; Lebestky et al., 2000). When ectopically expressed in the early mesoderm, *gcm* can induce expression of *Peroxidasin* (*Pxn*), which is a marker for macrophage cells. Misexpression of *gcm* in cells of the neural lineage also gives rise to a few cells that express hemocyte markers although most cells differentiate into glia (Bernardoni et al., 1997). In accordance with these findings, transcript

profiling of *gcm* misexpression embryos indicates that several genes encoding marker proteins for cells of the hemocyte lineage are differentially regulated.

In stage 15/16 embryos, differential expression levels are detected for *Pxn, serpent* (*srp*), and *the Scavenger receptor class C (type I)* gene (Pearson et al., 1995), all of which are expressed in hemocytes. Scavenger receptors play a crucial role in the phagocytosis of apoptotic cells and might also be able to mediate the direct recognition of microbial pathogens (Platt et al., 1998). It is noteworthy that the genes encoding Lysozyme B, Lysozyme C, Lysozyme D, and Lysozyme E are all upregulated by *gcm* misexpression in stage 11 embryos. These four closely related lysozyme genes, clustered at locus 61F on the third chromosome, function as part of a system of inducible antibacterial immunity (Daffre et al., 1994). These findings support the notion that the glial cell lineage and the hemocyte lineage, which gives rise to cells involved in defense and immunity, may be molecularly related (Bernardoni et al., 1997).

Analysis of spatial expression of candidate *gcm* downstream genes by in situ hybridization and immunocytochemistry

To complement the quantitative transcript profiling analysis with tissue-specific spatial expression data, in situ hybridization and immunostaining was carried out on a subset of the genes that are differentially regulated by *gcm* misexpression (Fig. 5). In all cases, the qualitative changes in tissue-specific gene expression revealed by in situ hybridization and immunocytochemistry reflect and confirm the changes in gene expression determined by transcript profiling.

Expression of the transcripts for *htl*, *scrt*, *bangles-and-beads* (*bnb*) and *elav* was examined by in situ hybridization. In stage 11 wild type embryos, the *htl* gene is expressed in a distinct set of neural precursors in the CNS (Fig. 5A). Outside of the CNS, *htl* is also expressed in elements of the mesodermal lineage (Shishido et al., 1997). Following targeted misexpression of *gcm* in cells of the neuroectoderm in stage 11 *sca-gcm* embryos, the expression of *htl* is expanded in the CNS region to include virtually all neural precursors (Fig. 5B). No changes in the expression of *htl* are seen outside of the CNS in these embryos. In stage 11 embryos, the pan-neural *scrt* gene is expressed in most or all neural precursors (Fig. 5C) (Roark et al., 1995). Following targeted misexpression of *gcm* in cells of the neuroectoderm in stage 11 *sca-*

gcm embryos, the expression of *scrt* is diminished in most of the neural precursors, but is still apparent in a subset of these cells (Fig. 5D).

In stage 15/16 wild type embryos, the *bnb* gene is expressed in lateral glial cells (Ng et al., 1989) (Fig. 5E,G). With the exception of a small group of cells near the anterior and posterior ends of the embryo, no other *bnb* expression is seen outside of the nervous system at this stage. In stage 15/16 *sca-gcm* embryos, the expression of *bnb* increases markedly and appears in virtually all of the cells of the nervous system (Fig. 5F,H). Expression of *bnb* outside of the nervous system does not appear to be influenced in these *sca-gcm* embryos. In stage 15/16 wild type embryos, the *elav* gene is expressed in all neurons (Fig. 5I). In stage 15/16 in *sca-gcm* embryos, expression of *elav* is strongly reduced, but is still visible in some neurons of the brain as well as in some of the neurons that occupy the ventral-most cell layer in the ventral nerve cord (Fig. 5J).

Given that transcript abundance is not always reflected on the protein level (Keene, 2001), expression of three further candidate *gcm* downstream genes, *ey*, *Ten-m*, and *wrapper* was investigated at the protein level by immunostaining in wild type and *sca-gcm* embryos. In stage 15/16 wild type embryos the EY protein is expressed in a segmentally reiterated subset of neurons in the CNS (Fig 5K). In stage 15/16 *sca-gcm* embryos the number of cells in the CNS that express the EY protein is dramatically reduced (Fig. 5L). In stage 15/16 wild type embryos, the TEN-M protein is expressed on the axons that make up the longitudinal and commissural tracts of the CNS (Fig. 5M). This well defined axonal expression pattern of TEN-M protein is virtually abolished in stage 15/16 *sca-gcm* embryos (Fig. 5N). The TEN-M protein is also expressed outside of the nervous system (Baumgartner et al., 1994), but there is no obvious change in this non-neuronal expression of TEN-M in *sca-gcm* as compared to wild type embryos. In stage 15/16 wild type embryos, the WRAPPER protein is expressed in the midline and in some of the lateral glial cells, as well as in glial cells that support the chordotonal sensory organs in the PNS (Fig 5O)(Noordermeer et al., 1998). In stage 15/16 *sca-gcm* embryos a substantial increase of WRAPPER expression is seen in the CNS (Fig. 5P).

To control for possible effects of transgene insertion or of differences in genetic background, we repeated the tissue-specific spatial expression analysis for all of the above mentioned genes on embryos that contain either only the *sca-GAL4* construct or only the *UAS-gcm*

constructs. In all cases, in situ hybridization and immunostaining results on these embryos were indistinguishable from results obtained on wild type embryos (data not shown).

To determine if genes, that are influenced by *gcm* gain-of-function, might be influenced in an inverse way in *gcm* loss-of-function mutants, we studied tissue-specific spatial expression data of the candidate *gcm* downstream genes *repo*, *bnb*, *wrapper*, *elav*, *ey* and *Ten-m* in *gcm* null mutants using in situ hybridization and immunostaining. Expression of the genes *repo*, *bnb* and *wrapper* is upregulated in *gcm* gain-of-function embryos. In stage 15/16 *gcm* null mutant embryos, the expression of the *repo* gene in lateral glial cells, which is seen in the wild type CNS, is strongly reduced (Fig. 5Q,R) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Comparable findings are obtained for *bnb*; the expression of the *bnb* gene in lateral glial cells, which is seen in the wild type CNS, disappears (Fig. 5Q,R). These findings contrast with stage 15/16 *sca-gcm* embryos, where the expression of *repo* and *bnb* appears in virtually all of the cells of the CNS. In stage 15/16 *gcm* null mutant embryos, the expression of *wrapper* in the lateral glial cells and in PNS glial cells (but not the midline glial cell expression), which is observed in wild type embryos, disappears (Fig. 5S,T). This contrasts with stage 15/16 *sca-gcm* embryos, where the expression of *wrapper* becomes more widespread in the CNS. Expression of the genes *elav*, *ey* and *Ten-m* is downregulated in *gcm* gain-of-function embryos. Expression of *elav* in *gcm* null mutants is seen in additional neuronal cells as compared to the wild type (data not shown) (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In contrast, the number of CNS cells that express either *ey* or *Ten-m* is not altered in *gcm* null mutants as compared to the wild type (data not shown). This is not unexpected since *ey* and *Ten-m* are not pan-neuronally expressed as is *elav* but are expressed only in a subset of neuronal cells in the wild type CNS (Baumgartner et al., 1994; Kammermeier et al., 2001).

Discussion

Candidate gene identification through genome-wide transcript imaging

By analyzing gene expression profiles following *gcm* misexpression in the embryonic CNS, genome-wide transcript images were obtained for two phases of glial development. The first transcript image reflects an embryonic CNS in which precursor cells that normally give rise to neurons have been genetically reprogrammed to give rise to glial cells. It was obtained at an early embryonic stage when the first glial-specific genes, such as the *repo* gene (which is a direct target of *gcm*), start to become expressed. This transcript image is therefore likely to identify genes that act in CNS precursors and are involved in the determination of glial versus neuronal cell lineages. Approximately 400 genes were found to be differentially expressed at this developmental stage, corresponding to 3% of the annotated genes in the fly genome. We posit that the genes that are differentially regulated at this early stage are either direct *gcm* target genes, such as *repo*, or among the initial set of downstream genes of *gcm*.

The second transcript image, obtained at a later embryonic stage when glial and neuronal cells are normally differentiated, reflects an embryonic CNS in which 80-90% of the normal number of neuronal cells have been genetically replaced by glial cells due to *gcm* action (Hosoya et al., 1995). This transcript image is therefore likely to identify genes that are involved in the maintenance of differentiated glial versus neuronal cells. Approximately 1300 genes were differentially expressed at this stage, corresponding to 9% of the annotated genes in the fly genome. We postulate that most of these differentially expressed genes are no longer direct or initial downstream targets of *gcm*, but are rather indirect downstream genes that act further along in the genetic cascade of *gcm* action.

The difference in total number of differentially expressed genes at the early stage versus the late stage is striking and, in qualitative terms, also holds for each of the major functional gene classes. Moreover, the overlap between the genes that are expressed at the two stages is restricted; only 93 (7%) of the 1259 genes that are differentially expressed at the late stage, are also differentially expressed at the early embryonic stage (Fig. 6). This suggests that the gene regulatory elements that control determination of glial cell fate are largely different from those required for maintenance of glial cell differentiation.

The expression profiles presented here derive from gain-of-function experiments in which the *gcm* gene is misexpressed in the embryonic CNS. A comparison of these findings with expression profiles derived from loss-of-function experiments involving *gcm* null mutants will be an important step in the further analysis of *gcm* downstream genes. However, in *gcm* null mutants only about 60 presumptive glial cells per segment are transformed into neurons; the 700 neurons of each segment are not affected. With the current sensitivity of oligonucleotide arrays, it is unlikely that significant measurements of gene expression changes in such a small number of cells can be obtained using whole-mount embryos. Thus, these complementary *gcm* loss-of-function experiments must await the development of single-cell isolation techniques for the embryonic nervous system of *Drosophila*.

Candidate genes implicated in the determination of glial versus neuronal cell lineage

Although *gcm* expression is necessary and sufficient to induce glial cell fate in and outside of the nervous system, it normally acts in glial precursors in the wild type (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Akiyama-Oda et al., 1998; Bernardoni et al., 1998). A current model of this action is that *gcm* controls the determination of glial cell fate in glial precursors by activating both genes that promote glial differentiation and genes that repress neuronal differentiation (Giesen et al., 1997). Application of this model to our experimental paradigm leads to the prediction that expression of glial precursor-specific genes should be upregulated and expression of neuron precursor-specific genes should be downregulated in stage 11 *sca-gcm* embryos. Our genome-wide expression data verifies this prediction.

We find upregulation of genes that are known to be expressed in glial precursor cells. Prominent among these is *repo*, which contains 11 GCM consensus binding sites in its upstream regulatory region and is the first identified direct target of *gcm* (Akiyama et al., 1996). Another upregulated gene that is first expressed in the CNS in glial precursors is *htl*, which encodes a FGF-receptor (Shishido et al., 1997). Downregulation is found for several genes that are known to be expressed in neuronal precursors. Prominent among these is the pan-neuronal gene *scratch* (*scrt*) which promotes neurogenesis and can induce additional neurons when ectopically expressed (Roark et al., 1995). Interestingly, we also observe downregulation for several genes that are involved in midline glial cell development such as *Egfr*, *vvl*, *en* and *D* (Condron et al., 1994; Anderson et al., 1995; Scholz et al., 1997; Soriano

and Russell, 1998). This suggests that midline glial development may be suppressed in *sca-gcm* embryos and might also explain the otherwise unexpected downregulation of *tramtrack* (*ttk*) and *pointed* (*pnt*), since these genes are not only expressed in lateral glial cells but also in midline glial cells (Granderath and Klambt, 1999).

In addition to genes that are known to be involved in the gliogenesis/neurogenesis decision, we find a large number of genes that have not previously been implicated in this aspect of CNS development. Indeed, for the majority of the known differentially regulated genes identified here, this report represents the first indication for an involvement in gliogenesis and/or neurogenesis. This is also the case for the annotated genes of unknown function, which have not been studied in any in vivo context, and which make up the majority of the differentially expressed genes identified.

The effects of targeted misexpression of *gcm* in stage 11 *sca-gcm* embryos appear to be restricted to cells of the neuroectoderm. Moreover, these effects manifest themselves primarily in altered gene expression in cells of the neuroectoderm. No morphological changes are seen in stage 11 *sca-gcm* embryos as compared to wild type, and non-specific side effects of *gcm* misexpression such as growth abnormalities, defective morphogenesis, or increased apoptosis are not observed in these embryos. We therefore assume that the observed differential gene expression specifically reflects activation or repression of *gcm* downstream genes. It is, nevertheless, unlikely that our study uncovers all of the genes that act downstream of *gcm* to induce glial cell fate. This is because our early transcript image is restricted to a specific time point in early gliogenesis development, and *gcm* may influence other targets at other stages. Moreover, the genetic overexpression of *gcm* may create an artificial situation in vivo, in which not all of the candidate downstream genes show changes in magnitude and direction of expression that correspond to their responses to *gcm* action under normal conditions. For example, whereas *gcm* expression in a mesodermal lineage induces genes involved in hemocyte cell development, overexpression of *gcm* in neuroectodermal cells causes a downregulation of the hemocyte marker genes *Pxn* and *srp*. (Downregulation of *Pxn* may, however, also be due to the fact that this gene is also expressed in the nervous system.) Finally, it is conceivable that some of the gene expression changes seen in *sca-gcm* embryos as compared to wild type are due to insertional effects of the transgenes or to differences in genetic background. While we find no evidence for such effects among the 10 *gcm* candidate genes that we characterized by in situ and immunocytochemical experiments, we can not rule

out such effects for all of the candidate genes identified in this report. In consequence, a full appreciation and verification of all of these candidate *gcm* downstream genes and a comprehensive understanding of their roles in determination of glial versus neuronal cell lineage will require a careful gene-by-gene analysis in mutant embryos. This also applies to the genes that are differentially expressed in stage 15/16 embryos.

Candidate genes implicated in the maintenance of differentiated glial versus neuronal cells

In stage 15/16 *sca-gcm* embryos most of the neurons in the embryonic nervous system are genetically replaced by glial cells, and differential gene expression in these embryos as compared to wild type embryos reflects this fact. While the transcript image obtained at this stage will, therefore, identify genes that are involved in the maintenance of differentiated glial versus neuronal cells, non-specific side effects of *gcm* misexpression on differential gene expression can not be ruled out. This is because the marked loss of neurons in stage 15/16 *sca-gcm* embryos results in morphological changes such as defective condensation of the CNS or reduction of peripheral innervation, and these morphological alterations may be accompanied by changes in gene expression.

Nevertheless, given that the strongest phenotype of stage 15/16 *sca-gcm* embryos is the gain of glial cells at the expense of neurons, we postulate that most of the observed differential gene expression at this stage is directly related to the replacement of differentiated neurons by differentiated glial cells. This is supported by the fact that a number of genes that are known to be expressed in differentiated neurons such as *elav*, *lark*, *Ten-m* and *CadN* are downregulated while genes that are expressed in differentiated glial cells such as *htl*, *wrapper* and *bnb* are upregulated in stage 15/16 *sca-gcm* embryos. In several cases, however, genes encoding markers for lateral or peripheral glia were not judged to be upregulated by our data analysis. For example, for the genes *repo*, *locomotion defects* (*loco*), and *gliotactin* (*gli*), the normalized expression levels, the fold change levels or the statistical significance levels were below our threshold filter values, so that these genes were not considered to be upregulated in our microarray experiments.

Cell fate determination is often controlled at the transcriptional level by key regulatory factors that are expressed transiently, whereas the gene expression patterns that they establish persist.

Maintenance of the transcriptional state in differentiated cells is then achieved by control elements involved in chromatin remodeling and modification (Francis and Kingston, 2001). Accordingly, in our analysis of stage 15/16 *sca-gcm* versus wild type embryos, we identified several differentially expressed genes that are involved in chromatin remodeling such as *Bap60, dalao*, *Snr1* and *ash2*. In specific glial lineages, the onset of differentiation is thought to require cell cycle progression (Akiyama-Oda et al., 2000). In our analysis, differential expression of genes encoding cell cycle regulators or proteins involved in chromatin condensation and segregation during mitosis was also observed. Examples of this are cyclin encoding genes such as *CycB*, *CycA, CycD* and *CycE,* which are differentially expressed in stage 15/16 embryos. The differential expression of these genes following *gcm* misexpression provides further support for the general notion that cell cycle regulators are key elements in cellular differentiation processes (Ohnuma et al., 2001).

In summary, this study combines in vivo transgenic analysis with genome-wide expression analysis based on oligonucleotide arrays to identify genes that are downstream of *gcm*, a key transcriptional control element in gliogenesis. The results of this study should be helpful in obtaining a comprehensive view of the molecular mechanisms of cell fate specification and cell type maintenance in the developing nervous system.

Acknowledgments

We thank A. Roth, M. Tessier, M. Wilhelm-Seiler, C. Broger, M. Neeb, R. Brem, A. Giangrande, K. Takizawa, B. W. Jones, K. J. Schmid, M. Affolter and his group, A. Ebner, L. Michaut, A. Tomlinson, S. Baumgartner, U. Walldorf, R.A.H. White, J. N. Noordermeer, H. Montalta-He, the Bloomington Stock Center for technical support, reagents and flies and M. Müller, F. Hirth, and M. Götz for helpful comments on the manuscript.

TABLE

Table 1. Differential gene expression in functional classes following *gcm* misexpression

Molecular Function	Number of transcripts	
	stage 11	**stage 15/16**
Nucleic acid binding	44	96
DNA binding	33	64
Transcription factor	26	38
RNA binding	6	27
Translation factor	5	4
Ribonucleoprotein	0	1
Cell cycle regulator	0	10
Chaperone	7	13
Motor protein	4	7
Defense/immunity protein	4	3
Enzyme	78	249
Kinase/Phosphatase	13	59
Enzyme activator	0	3
Enzyme inhibitor	3	7
Apoptosis regulator	0	2
Signal transducer	12	50
Cell adhesion	4	19
Structural protein	3	39
Transporter	25	51
Ligand binding or carrier	13	63
Antioxidant	1	2
Tumor suppressor	1	0
Function unknown	218	645
Total	**417**	**1259**

FIGURES

Figure 1. Targeted misexpression of *gcm* results in gain of glial cells at the expense of neuronal cells.

(A,B) In situ hybridization of stage 10 embryos shows *gcm* expression in the wild type (A) and in *sca-gcm* embryos (B); lateral views, anterior towards the left. In the wild type, small clusters of cells in the neuroectoderm express *gcm*; in *sca-gcm* embryos, all cells of the neuroectoderm express *gcm*. (C,D) Immunostaining with anti-REPO in the wild type (C) and in *sca-gcm* embryos (D); laser confocal microscopy of stage 11, ventral views of the VNC, anterior is to the left. In the wild type, single *gcm*-expressing glial precursors in each hemisegment express the *repo* gene. In *sca-gcm* embryos, virtually all of the neuronal and glial precursor cells are REPO positive. (E,F) Double immunostaining with anti-REPO (green), and anti-HRP (red) in the wild type (E) and in *sca-gcm* embryos (F); laser confocal microscopy of stage 15/16 embryos. In the wild type, neurons and glial cells are differentiated and correctly positioned, and all lateral glial cells express *repo*. In *sca-gcm* embryos, 80%-90% of the cells in the CNS express *repo*.

Figure 2. Changes in transcript levels of the genes encoding transcription factors following *gcm* misexpression.

Bars represent the fold changes in gene expression levels between wild type embryos and *sca-gcm* embryos. Positive values indicate that the relative expression level of a gene is increased (upregulation) and negative values indicate a decrease (downregulation). Normalized average difference (Avg Diff) values are given for the wild type condition as follows: yellow bars represent Avg Diff <100, orange bars represent Avg Diff ranging from 100-1000, and red bars represent Avg Diff >1000.

Figure 3. Changes in transcript levels for the genes encoding protein kinases and phosphatases following *gcm* misexpression.
Data are presented as in figure 2.

Figure 4. Changes in transcript levels for the genes encoding DNA/chromatin binding proteins, cell cycle regulators or cell adhesion molecules following *gcm* misexpression.
(A) Genes encoding DNA/chromatin binding proteins. (B) Genes encoding cell cycle regulators. (C) Genes encoding cell adhesion molecules. Data are presented as in figure 2.

Figure 5. Spatial expression of selected candidate *gcm* downstream genes by in situ hybridization and immunocytochemistry.

Whole mount in situ hybridization (A-J,Q,R) and immunostaining (K-T) show expression of differentially regulated genes in wild type, *sca-gcm* and *gcm* mutant embryos. Ventral views of stage 11 (A-D) and stage 15/16 (E,F,I,J,K-P) embryos and lateral views of stage 15/16 embryos (G,H,Q-T), anterior is to the left. Fold changes and p-values are indicated on the right. (A,B) Expression of *htl* in stage 11 wild type embryos is visible in a distinct set of neural precursors; in sca-*gcm* embryos, *htl* is expressed throughout the neurogenic region. (C,D) In stage 11 embryos, the *scrt* gene is expressed in neural precursors; in stage 11 *sca-gcm* embryos, the expression of *scrt* is diminished in most of the neural precursors, but is still apparent in a subset of these cells. (E-H) In stage 15/16 wild type embryos, *bnb* gene is expressed in lateral glial cells; in stage 15/16 *sca-gcm* embryos, the expression of *bnb* increases markedly and appears virtually in all of the cells of the nervous system. (I,J) In stage 15/16 wild type embryos, the *elav* gene is expressed in all neurons; in stage 15/16 in *sca-gcm* embryos, expression of *elav* is strongly reduced in most of the neurons. (K,L) In stage 15/16 wild type embryos the EY protein is expressed in a segmentally reiterated subset of neurons in the CNS; in stage 15/16 *sca-gcm* embryos the number of EY expressing cells in the CNS is dramatically reduced. (M,N) In stage 15/16 wild type embryos, the TEN-M protein is expressed on the axons that make up the longitudinal and commissural tracts of the CNS; this axonal expression of TEN-M is virtually abolished in stage 15/16 *sca-gcm* embryos. (O,P) In stage 15/16 wild type embryos, the WRAPPER protein is expressed in midline glial cells, in some lateral glial cells and in glial cells supporting the chordotonal sensory organs; this expression has spread to the complete CNS region in stage 15/16 *sca-gcm* embryos. (Q,R) In late stage embryos REPO (brown) is expressed in all and *bnb* (blue) is expressed in a subset of lateral glial cells; in *gcm* mutants REPO expression is reduced to a few cells, and *bnb* expression is completely absent in the CNS. (S,T) In late stage embryos WRAPPER is expressed in midline glial cells, in some lateral glial cells and in glial cells supporting chordotonal sensory organs (arrowheads); in *gcm* mutant embryos WRAPPER expression in lateral glia (CNS) and in chordotonal sensory organs (PNS) is absent whereas expression in midline glial cells remains.

Figure 6. Changes in transcript levels of the genes with differential expression in both early and late embryonic stages following *gcm* misexpression.

93 genes show significant changes in expression levels in response to *gcm* misexpression at stage 11 (yellow) as well as at stage 15/16 (red). Bars represent the fold changes in gene expression levels between wild type embryos and *sca-gcm* embryos. Positive values indicate that the relative expression level of a gene is increased (upregulation) and negative values indicate a decrease (downregulation).

4. Isolation of genetically labeled cells by magnetic cell sorting from the neuroectoderm of *Drosophila* embryos improves genome-wide microarray analysis of *glial cells missing* downstream genes

Haiqiong Montalta-He[1], Yun Fan[1], Boris Egger[1], Ulrich Certa[2] and Heinrich Reichert[1]

[1] Institute of Zoology, Biocenter/Pharmacenter, Klingelbergstrasse 50, University of Basel, CH-4056 Basel, Switzerland
[2] Roche Genetics Pharmaceuticals Division, F.Hoffmann-La Roche, Ltd., CH-4070 Basel, Switzerland

Running Title: microarray analysis, magnetic cell sorting in *Drosophila*

Key Words: magnetic cell separation, *gcm/glide*, *Drosophila*, gene expression profiling

Summary

A combination of genetic labeling and magnetic cell sorting was used for isolating neuroectodermal cells from *Drosophila* embryos for microarray analysis of *gcm* action in neurogliogenesis. The *GAL4-UAS* system was used to direct expression of mCD8-GFP, a molecular label suitable for magnetic cell isolation, exclusively to the neuroectoderm of stage 11 embryos. Labeled cells were then dissociated and separated using magnetic cell sorting techniques, which permitted a high rate of purification of viable cells from the neuroectoderm as assayed by both cellular and molecular methods. Using this cell separation technique in combination with full genome microarrays, differential gene expression was analyzed in wild type embryos versus embryos in which *gcm* was misexpressed throughout the neuroectoderm. For comparison, we used the same microarrays to analyze differential gene expression in the same two sets of embryos as whole mounts. In microarray experiments involving sorted cells, 76 genes were identified as differentially expressed following *gcm* misexpression. This contrasted with the results of the whole mount-based experiments, in which 242 genes were judged as differentially expressed following *gcm* misexpression. Moreover, validation studies by in situ hybridization of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed a rate of confirmation of more than 80%. These experiments imply that reduction of cell heterogeneity through cell sorting techniques leads to a marked increase in the ability of microarrays to reveal differential gene expression in the developing nervous system.

Introduction

The formation of a functional nervous system requires the correct specification of a large number of different cell types. These cell types fall into two major categories, neurons and glial cells (Jones, 2001). Accordingly, an important issue in developmental neurobiology is to understand how this diversity is generated in the nervous system. *Drosophila* has proved to be an excellent genetic model to study the mechanisms involved in neurogliogenesis, and significant progress has been made in understanding the mechanisms underlying neuron-glia fate switch, symmetric-asymmetric division of the multipotent precursors, and sublineage specification (Bossing et al., 1996; Schmidt et al., 1997; Schmid et al., 1999; Anderson, 2001).

In *Drosophila*, the gene *glial cell deficient/glial cells missing* (*gcm*) is the master regulator of glial cell fate determination. It encodes a transcription factor that is transiently expressed in glial precursors in the neuroectoderm (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). In the *gcm* mutant, cells that normally develop into glia enter a neuronal differentiation pathway leading to a loss of glia and a gain of neurons. By contrast, misexpression of *gcm* in neural progenitors results in an increase of glial cells at the expense of neurons (Akiyama-Oda et al., 1998; Bernardoni et al., 1998; Jones, 2001). The molecular mechanisms of *gcm* action in *Drosophila* are thought to be mediated through the regulation of *gcm* downstream target genes. However, until recently, molecular genetic analyses have identified only few genes as *gcm* targets that are involved in gliogenesis (Klambt, 1993; Campbell et al., 1994; Klaes et al., 1994; Xiong and Montell, 1995; Granderath et al., 1999).

In order to identify additional *gcm* target genes, two genome-wide microarray experiments have been carried out recently using whole mount embryos in which *gcm* was misexpressed genetically throughout the entire neuroectoderm (Egger et al., 2002; Freeman et al., 2003). Both studies reported the identification of a large number of differentially expressed candidate genes following *gcm* misexpression in embryos as compared to wild type-like control embryos. However, changes of gene expression could be validated by in situ hybridization or immunostaining for only a very limited number of these candidate genes. Indeed, based on the low level of validation, an estimate of the number of false positive results in whole embryo microarray studies of this type has been given at 88% (Freeman et al., 2003). In our case

(Egger et al., 2002), a false positive rate of about 70% was revealed by further in situ hybridization studies (Fan, unpublished data). Clearly, such a high level of false positives results would hinder the further application of microarray technology to studies of neurogliogenesis in *Drosophila*.

The low level of validation attained in conventional microarray experiments is a general problem, and one of the major reasons for this drawback appears to be the complexity of the tissue used for the microarray experiments. For the microarray experiments mentioned above, the whole embryos rather than the neuroectoderms of the embryos had been involved which certainly contributed to the high false positives. The high complexity of the tissue samples used creates a signal-to-noise problem for the specific detection of gene expression in a given microarray experiment (Barlow and Lockhart, 2002; Griffin et al., 2003; Henry et al., 2003). One way to solve problems of tissue heterogeneity is to reduce as much as possible the irrelevant tissues. This can be achieved by dissecting the part of the organisms interested or purifying specific cell types from complex tissue. There have been several successful examples of microarray experiments based on purification of specific cell types; these include the application of Laser Captured Microdissection (LCM), Fluorescent Associated Cell Separation (FACS), single cell transcript profiling or mRNA-tagging (Bryant et al., 1999; Mills et al., 2001; Roy et al., 2002; Luzzi et al., 2003; Tietjen et al., 2003). Thus, access to a homogeneous population of specific cell types facilitates the application of microarray analysis in developmental biology.

In this report we adapted the well established method of magnetic cell separation (MACS) (Safarik and Safarikova, 1999) to isolate neuroectodermal cells from *Drosophila* embryos for microarray analysis of *gcm* action in neurogliogenesis. For this purpose, neuroectodermal cells were genetically labeled with a transmembrane fusion protein consisting of murine CD8 fused with GFP (mCD8-GFP); following cell dissociation, mCD8-positive neuroectodermal cells were incubated with magnetic microbeads coupled with anti-mCD8 antibody and were subsequently enriched by magnetic sorting. Using this cell separation technique in combination with full genome microarrays, we analyzed differential gene expression in wild type embryos versus embryos in which *gcm* was misexpressed throughout the neuroectoderm. For comparison, we used the same microarrays to analyze differential gene expression in the same two sets of embryos as whole mounts, i.e. without cell separation. In microarray experiments involving sorted cells, 76 genes were identified as differentially expressed

following *gcm* misexpression. This contrasted with the results of the whole mount-based experiments, in which 242 genes were judged as differentially expressed following *gcm* misexpression. Moreover, validation studies by in situ hybridization of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed high rates of confirmation. Taken together, our experiments imply that reduction of cell heterogeneity through cell sorting techniques leads to a marked increase in the ability of microarrays to reveal differential gene expression in the developing nervous system.

Material and methods

Flies

Drosophila melanogaster stocks were raised on standard cornmeal/yeast/agar medium at 25°C. To label the embryonic neuroectoderm, virgin females from *scabrous-GAL4* (*sca-GAL4*) (Klaes et al., 1994) were crossed to w^{1118};;*UAS-mCD8-GFP* males. Previous studies indicated that there is no detectable toxicity due to overexpression of the mCD8-GFP fusion protein in *Drosophila* (Lee and Luo, 1999). For ectopic expression of *gcm*, *sca-GAL4* virgins were crossed to w^{1118};;*UAS-mCD8-GFP,UAS-gcm* (Bernardoni et al., 1997). After a 1 hr pre-collection, embryos were collected in parallel for 1 hr and staged to 6-7 hrs AEL (late stage 11). Stages are according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

Embryo dissociation

Embryos were dechorionated in 3-4% chlorax for 4min, collected on a mesh and rinsed thoroughly with water. After incubation with 60µg/ml Proteinase K (Invitrogen, 20mg/ml) in phosphate buffered saline (PBS) for 2min, embryos were dried and transferred into a 15ml polypropylene screw-cap tube (Falcon) containing 1ml of 10x Trypsin-EDTA (GIBCO, Cat# 35400-027). The embryos were homogenized by passing ~20 times through a 21G needle. After adding 4ml PBS into the tube, the tube was fixed on a shaker and incubated for 30min at 25ºC at 800rpm. Finally, the homogenate was filtered through a cell strainer (40µm, Falcon) to remove tissue clumps. Cells were pelleted at 1500rpm, 4°C for 5min in a tabletop centrifuge. After resuspension in 0.5ml of MACS buffer (1xPBS, 0.5% BSA and 2mM EDTA), cells were incubated with Hoechst 333342 (2µg/ml final concentration; Molecular Probes) and/or propidium iodide (10µg/ml final concentration; Molecular Probes) for 30min

at room temperature to allow dyes to equilibrate. Flow cytometer analysis was performed in a Becton Dickinson FACSCalibur flow cytometer.

Magnetic Cell Sorting (MACS)

Cell concentration was determined using haemacytometer before cells were resuspended in 90µl of MACS buffer per 10^7 total cells. 10µl of MACS CD8a(Ly-2) Microbeads (Miltenyi Biotec, Cat# 130-090-401) was added to every 10^7 cells. Free microbeads were washed away by adding 10-20x labeling volume of MACS buffer and centrifuging at 1500rpm for 5min. Cells were resuspended in 0.5ml MACS buffer and loaded onto a prepared MS+/RS+ type column (Miltenyi Biotec, Cat# 130-042-201) in the magnetic field of an octoMACS separator (Miltenyi Biotec, Cat# 130-042-109). Non-labeled cells flowed through the column while labeled cell were retained in the column. After washing the column four times with MACS buffer, retained cells were flushed out in 1ml MACS buffer using the plunger supplied with the column. Cells were pelleted and stored in liquid nitrogen before total RNA isolation.

Real Time Reverse Transcription Polymerase Chain Reaction

For real time RT-PCR experiments, 300ng poly $(A)^+$ RNA was isolated (mRNA isolation kit; Roche Diagnostics) and reverse transcribed with AMV-RT and random hexamers (RT-PCR kit; Roche Diagnostics). PCR was performed with 100pg template DNA and gene-specific primers (Seq Web, Winsconsin Package Version 10.0, GCG) on a light cycler (LightCycler, Roche Diagnostics). Continuous fluorescence observation of amplifying DNA was possible using SYBR Green I (Roche Diagnostics). After cycling, a melting curve was produced by slow denaturation of the PCR end products to validate the specificity of amplification. To compare the relative amounts of PCR products we monitored the amplification profile on a graph, displaying the log of the fluorescence intensity against the number of cycles. Relative fold changes for a given gene under both conditions (sorted *vs.* flowthrough) were calculated using the fit point method (LightCycler Manufacturer, Roche).

RNA isolation, target preparation and hybridization

Total RNA from sorted cells and embryos was extracted using a Mini RNA Isolation Kit (Zymo Research, Cat# R1005) and was eluted with RNase-free water. Quality and quantity of the RNA samples were assessed using a RNA 6000 NANO Chip (Agilent Technologies, Cat# 5065-4476). Because of the small amount of total RNA derived from the MACS sorted cells, we used a commercial Microarray Target Amplification Kit (Roche, cat# 3 310 191) to

synthesize target cRNA; this involved PCR-amplification of cDNA after normal retro-transcription. Target cRNA preparation from the embryos was as previously described (Leemans et al., 2001). In both cases, 20µg of biotinylated antisense cRNA were ultimately hybridized to the arrays according to standard protocol (Montalta-He et al., 2002).

Oligonucleotide Arrays

For expression profiling, DrosGenome1, a high density oligonucleotide array (Affymetrix, cat# 900 335) was used. This array was based on the Release 1.0 of the *Drosophila* genome. (Sequences were downloaded from the Flybase database on August 25th, 2000.) Sequences on the array represented more than 13,500 predicted transcripts as well as different control genes. Each sequence is represented on the array by a set of 14 oligonucleotide probes of matching sequence and 14 oligonucleotide probes with a single nucleotide mismatch. The signal intensity is calculated by an algorithm based on the perfect match hybridization signal and on the mismatch hybridization signal, and is proportional to the abundance of a given transcript (Rajagopalan, 2003). Four replicates were performed for each experimental condition.

Data analysis

Data acquisition and processing by RACE-A was as described elsewhere (Montalta-He et al., 2002). For quantification of relative transcript abundance, the value of signal intensity was used. All arrays were normalized against the mean of the total sums of signal intensity values. For differential transcript imaging, only transcripts that showed an expression level fold change (FC) $\geq$ 2.0 or $\leq$ -2.0 at significance values of $p \leq 0.01$ (unpaired t-test) were considered to be differentially expressed. The complete list of the microarray expression data involved can be accessed at http://www.ncbi.nlm.nih.gov/geo/. Accession number: GSE612 (sorted experiment) and GSE613 (whole mount experiment).

Synthesis of RNA probes from PCR products

Primers for in situ hybridization probe synthesis were designed according to the coding region of the gene studied. A T3 promoter sequence, 5' ATTAACCCTC ACTAAAGGGAGA 3', was added to the 5 prime of the 3 prime end primer. Normal PCR reaction was performed using cDNA from the stage 11 embryos as the template (Kain et al., 1991). RNA probes were prepared according to the standard protocol.

In situ hybridization and immunocytochemistry

In situ hybridization was carried out according to Tautz and Pfeifle (Tautz and Pfeifle; 1989). Embryos were mounted in Canada balsam (Serva), viewed on a Zeiss Axioskop microscope with differential interference contrast optics and photographed with a Prog/Res/3008 digital camera (Kontron). Immunocytochemical experiments were carried out as described previously (Therianos et al., 1995). The primary antibodies were rat anti-RK2/REPO diluted 1:1000 (Campbell et al., 1994). For fluorescent labeling, rat anti-mouse CD8-FITC (Miltenyi Biotec) was used 1:10 and secondary antibodies were Alexa568 and Alexa488 conjugated and diluted 1:150 (Molecular Probes). Fluorescently labeled embryos were viewed with a Leica TCS SP confocal microscope.

Results

Labeling of the embryonic neuroectoderm and targeted misexpression of *gcm*

For a genome-wide identification of genes that are either direct *gcm* target genes or among the initial set of downstream genes of *gcm*, we studied differential gene expression at embryonic stage 11. At this stage, the first glial marker, the direct *gcm* target gene *repo,* is expressed in the neuroectoderm. Cells in the neuroectoderm of stage 11 embryos were labeled genetically with a transmembrane protein consisting of murine CD8 fused with GFP. This was achieved by crossing a *sca-Gal4* enhancer trap line (Klaes et al., 1994) with a *UAS-mCD8-GFP* line (Lee and Luo, 1999), and resulted in uniform labeling of the cell surface of all cells in the neuroectoderm. This was referred to as the wild type-like situation. An example of the extent of this type of neuroectodermal labeling by mCD8-GFP in stage 11 embryos is shown in figure 1A; localization of intense staining at the surface of the cells is very clear. No homologs of mCD8 or GFP exist in *Drosophila*, thus the only cells in the embryo that express mCD8 or GFP are those targeted by the GAL4-UAS system. In order to misexpress *gcm* in the labeled embryonic neuroectoderm of stage 11 embryos, the same *sca-GAL4* line was crossed with a recombinant *UAS-mCD8-GFP, UAS-gcm* line of which the neuroectoderm is also labeled by mCD8-GFP (Fig. 1B). This was referred to as the *gcm* misexpression situation.

With the exception of altered gene expression in cells of the neuroectoderm, no obvious morphological changes were seen in the stage 11 wild type-like or *gcm*-misexpression embryos.

In the wild type-like situation, endogenous *gcm* expression was seen in two small groups of neuroectodermal cells in each hemisegment during stages 10-11 (Fig. 1C). At stage 11, a single *gcm*-expressing neural precursor delaminated from each of these groups and gave rise to cells expressing the glia-specific *repo* gene, a direct target of *gcm* (Fig. 1E) (Hosoya et al., 1995; Jones et al., 1995). In contrast, in the targeted *gcm* misexpression situation, all of the cells in the neuroectoderm expressed *gcm* at stages 10-11 (Fig. 1D). In consequence, many more neural precursor cells expressed the glia-specific *repo* gene at stage 11 (Fig. 1F) (Egger et al., 2002).

MACS allows efficient recovery of mCD8-labeled cells from dissociated embryonic neuroectoderm

Cells were dissociated from stage 11 embryos by homogenization and treatment with trypsin followed by filtration (see Materials and methods). To assess the extent of cell dissociation and the rate of cell survival, cells were stained with Hoechst 33342 for visualization of nuclei and with propidium iodide, which is excluded from cells with intact membranes and hence stains the dead cells. Examination of dissociated cell preparations stained with Hoechst 33342 showed that the majority of the stained material corresponded to single cells (Fig. 2A,B). Some small clusters of non-dissociated cells were also observed. Examination of dissociated cell preparations stained with propidium iodide showed that only very few cells were stained with propidium iodide (Fig. 2C). For quantification of this, propidium iodide stained preparations were analyzed by a flow cytometer; this analysis indicated that over 90% of dissociated cells were viable judged by their low levels of propidium iodide staining (Fig. 2D). Based on the GFP signal from the flow cytometer, around 20-30% of the cell population is genetically labeled (data not shown).

After cell dissociation, microbeads coupled with anti-mCD8 antibody were incubated with the concentrated cell solution and then applied to a separation column in a magnetic field to enrich for mCD8 expressing cells (see Material and methods). According to the MACS

protocol used, mCD8-positive neuroectodermal cells were expected to be selectively retained in the column while the mCD8-negative non-neuroectodermal cells were expected to flow through. To assay the degree of cell purification obtained by the MACS procedure, both the mCD8-positive neuroectodermal cells (referred to as sorted fraction) and the mCD8-negative cells (referred to as flowthrough fraction) were characterized by studying GFP fluorescence (cell labeling was achieved with a mCD8-GFP fusion protein). As expected, only a few cells in the flowthrough fraction were GFP positive judged by fluorescence microscopy (Fig. 3A,B). In contrast, the majority of the cells in the sorted fraction appeared as GFP positive (Fig. 3C,D). For quantification and control of the degree of GFP labeling of cells in the sorted fraction, a flow cytometry analysis was carried out for each experiment performed in this study. In all cases, flow cytometry analysis indicated a >60% purity of GFP-positive cells, and in some cases values as high as 85% purity were obtained (Fig. 3E). Compared with the cell population before sorting, MACS leads to a 3-4 fold enrichment of GFP-positive cells. The overall morphology of the cells in both fractions appeared to be normal. Cell separation and purification rates obtained from wild type-like embryos and from *gcm* overexpression embryos were not significantly different. Taken together, this indicates that a marked enrichment of the mCD8-positive neuroectodermal cells was obtained through cell dissociation and magnetic cell separation.

For an independent molecular confirmation of the enrichment efficiency of the cell sorting procedure, real-time RT-PCR was performed on cDNA prepared from the sorted fraction versus the flowthrough fraction for three genes: *sca* was chosen as a positive control because sorted cells were derived from the endogenous *sca* domain; *cg9232* gene, which is expressed in the embryonic endoderm and posterior/anterior midgut primordium hence should not be enriched in the sorted neuroectodermal cells, was for negative control; finally, the ubiquitously expressed *rp49* (*ribosomal protein 49*) gene was selected for base line control. 4 independent replicates, derived from two different cDNA preparations were carried out. As expected, *rp49* had similar expression levels in both fractions. The *sca* gene was enriched 5.7-fold in the sorted fraction as compared to the flowthrough fraction; this indicates a marked enrichment of endogenous *sca*-expressing neuroectodermal cells (which are also labeled transgenically with *sca-GAL4/UAS-mCD8-GFP*) in the sorted fraction. Conversely, the *cg9232* gene was enriched 4.6-fold in the flowthrough fraction as compared to the sorted fraction. These results confirm, at the molecular level, that it is possible to markedly enrich

for neuroectodermal cells labeled with mCD8-GFP from the embryos using the magnetic cell separation procedure.

Overview of gene expression profiling following *gcm* overexpression in the embryonic neuroectoderm

Analyses of differential gene expression in the *gcm* misexpression versus wild type-like situations were carried out using full-genome high density oligonucleotide arrays. Differential gene expression was determined by transcript profiling of sorted cells derived from *gcm* misexpression embryos as compared to transcript profiling of sorted cells derived from wild type-like embryos. Four replicates were performed for each experimental condition. These experiments identified 76 genes as differentially regulated following *gcm* overexpression. The same number of transcripts had upregulated (n=38) and downregulated (38) expression levels. All 76 genes were classifed based on molecular function according to Gene Ontology (Ashburner et al., 2000) (Table 1). Strikingly, but not surprisingly, the majority of the differentially regulated genes (54%) were of currently unknown function. The two functional classes with the largest number of differentially regulated transcripts were enzymes (15) and nucleic acid binding (12), including 10 transcription factors.

The relatively small number of 76 genes identified as differentially regulated following *sca-GAL4/UAS-gcm* mediated misexpression of *gcm* in the neuroectoderm obtained in this cell sorting-based microarray experiment contrasts with the larger numbers of differentially regulated genes obtained in comparable whole mount-based microarray experiments involving *gcm* misexpression (Egger et al., 2002; Freeman et al., 2003). To determine if these differences in gene numbers might be due to the different cell-sorting versus whole mount situations, we repeated out transcript profiling experiments on whole mount embryos identical to those used for cell sorting (*gcm* misexpression versus wild type-like).

In the transcript profiling experiments involving whole mount embryos, 242 transcripts were judged to be differentially regulated by *gcm* overexpression as compared to wild type. Approximately the same number of transcripts had increased (n=116) and decreased (n=126) expression levels. Classification of these differentially expressed genes (Table 1) showed that the majority of genes (51%) were of unknown function, and the two functional classes with the largest number of transcripts were again enzymes (65) and nucleic acid binding (18),

including 14 transcription factors. Only 13 of the 242 transcripts judged to be differentially expressed in the whole mount-based experiment were also found as differentially expressed in the cell sorting-based experiment.

A comparison of the results obtained in the cell sorting-based experiments versus those obtained under otherwise identical conditions in whole mount-based experiments, shows clearly that much fewer genes were judged to be differentially regulated following cell sorting. It seems likely, that this is due to the use of more homogeneous target tissue in the cell sorting experiments.

Quantitative transcript profiling of differentially expressed genes in cell sorting-based experiments

Figure 4 shows the differentially regulated genes identified by transcript profiling of sorted cells derived from *gcm* misexpression embryos versus wild type-like embryos. It presents a quantitative representation of the change in expression levels for these gene transcripts. The gene with the highest increase in expression level (12.9-fold) was *cg9541*, which has been predicted to encode an adenylate kinase. Increases in expression level above 10-fold were also observed for *cg3132*, which encodes a beta-galactosidase. There were 4 transcripts which all encode proteins of unknown function and showed increases in the 5-10-fold range, namely *cg12910*, *cg6218*, *cg5822* and *cg12641*. The first three have been shown to be expressed in glial cells and be potential *gcm* regulated genes (Freeman et al., 2003). The majority of the remaining upregulated transcripts have increases in the range of 2-5 folds.

None of the genes with decreased expression levels have decrease levels below –5 fold. The gene with the largest decrease in expression level (-4.9-fold) was *cg14830*; its function is currently unknown. Interestingly, among the genes with downregulated expression levels are several genes, which have been shown to act in neuronal cells or neuronal precursors. These are *al*, *ey*, *ewg*, *nrm*, *scrt* and *ftz* (Doe et al., 1988; DeSimone and White, 1993; Roark et al., 1995; Kammermeier et al., 2001; Egger et al., 2002). The downregulation of these genes is in accordance with the model of GCM action during gliogenesis that *gcm* suppresses genes functioning in the neuronal cell lineages (Giesen et al., 1997).

Validation of results of cell sorting experiments by in situ hybridization in embryonic CNS

Can the differential expression of genes that result from microarray analysis of cell sorted *gcm* misexpression embryos be confirmed by tissue-specific spatial expression studies? To address this question, we focused on the set of 17 genes among the genes of the highest increase in expression levels in the sorting-based experiment. These genes are highlighted in bold in figure 4. Among these 17 genes, 9 have been shown by in situ hybridization to be differentially expressed following *gcm* overexpression in previous reports (Fig. 4) (Freeman et al., 2003). The remaining 8 genes were studied by in situ hybridization using the same transgenic lines as those employed for microarray analysis. Among them, 5 showed clear differential expression patterns in *gcm* overexpression embryos as compared to wild type-like embryos (Fig. 5 and see below for in situ data). Thus, among the 17 genes that we considered and that were studied by in situ hybridization, a total of 14 (82%) were validated by in situ hybridization as differentially expressed in embryonic tissue following *gcm* misexpression.

Interestingly, 7 of these 14 validated genes from the cell sorting-based microarray experiment were also found among the genes judged to be differentially expressed in the whole mount-based microarray experiment (Table 2). These are actually all the upregulated genes in the overlap of sorted and whole mount experiment. This suggests that genes judged as differentially expressed in both the cell-sorted microarray experiment and in the whole mount-based microarray experiment might have high rate of validity. To investigate this, we performed in situ hybridization studies for another 4 genes judged as downregulated in both experiments (Table 2). Among these 4 genes, 3 showed clear differential expression patterns in *gcm* overexpression embryos as compared to wild type-like embryos. Thus, among the 13 genes that showed differential expression in both microarray studies, 11 have been studied by in situ hybridization, and 10 of these (91%) were validated by in situ hybridization as differentially expressed in embryonic tissue following *gcm* misexpression (Table 2, see below for in situ data).

Taken together, the in situ hybridization studies carried out here validated 8 new candidate *gcm* downstream genes. The in situ expression patterns of these 8 genes are shown for the wild type-like versus the *gcm* misexpression situation of stage 11 embryos in figure 5. In the case of *cg9541*, *gcm* misexpression resulted in an increased expression of the gene in specific,

segmentally repeated domains of the ventral neuroectoderm (Fig. 5A,B). A similar situation was observed for *cg15307*, where *gcm* misexpression caused an increase in gene expression in segmentally reiterated domains of the ventral neuroectoderm as well as in specific cephalic regions (Fig. 5C,D). For *cg12641* a comparable increase in expression in the segmentally repeated domains of ventral neuroectoderm was seen following *gcm* misexpression, however, this increase was not as strong as those observed for *cg9541* and *cg15307* (Fig. 5E,F). In contrast to the relatively broad expression domains of these three genes in the wild type-like situation, the expression domains of the genes *cg3132* and *cg6560* were more localized to specific groups of neuroectodermal cells in the wild type-like situation (Fig. 5G,I). Accordingly for these two genes, *gcm* misexpression resulted in an increase of expression localized to these groups of neuroectodermal cells (Fig. 5H,J). The genes *nerfin-1* and *cg17649* were expressed in a comparable set of neuroectodermal cells in the wild type-like situation (Fig. 5K,M). For *nerfin-1*, these neuroectodermal cells have been identified as neuroblasts and ganglion mother cells (Stivers et al., 2000). Following *gcm* misexpression throughout the neuroectoderm, the expression of nerfin-1 and *cg17649* was strongly reduced or even abolished in some of these cells (Fig. 5L,N). For the *al* gene, the wild type-like expression at stage 11 has been characterized as restricted to a segmentally repeated pattern of three thoracic and eight abdominal lateral patches of which the eighth spot conceivably labels the anterior lateral sense organs (Fig. 5O) (Schneitz et al., 1993). Misexpression of *gcm* in the neuroectoderm appeared to repress the expression of *al* in the abdominal patches (Fig. 5P).

Discussion

Genetic labeling coupled with magnetic cell separation leads to efficient isolation of viable cells from genetically specified embryonic domains

Microarray studies involving whole mount tissues are inherently complicated by the diversity of cell populations. This problem is more prominent for studies of the nervous system where cells of interest may comprise only a fraction of the entire tissue studied, anatomical divisions between regions of the nervous system are often unclear, and precisely controlled, artifact-free dissections are difficult (Barlow and Lockhart, 2002; Griffin et al., 2003). In consequence, averaging expression levels of entire tissue regions, or of entire embryos, may minimize or conceal even large expression changes that occur in small subpopulations of cells. This problem is aggravated in studies of neuronal development due to the small size of embryonic nervous system and the difficulty in identifying the subpopulations of interest for dissection in embryos. In order to overcome this experimental obstacle, methods are needed that allow the isolation of part of the organism interested or specific cell subpopulations from whole mount tissues.

In this study we have used a combination of genetic labeling techniques and magnetic cell sorting for isolating neuroectodermal cells from *Drosophila* embryos for microarray analysis. The high spatiotemporal specificity of the *GAL4-UAS* system was used to direct expression of mCD8-GFP, a molecular label suitable for magnetic cell isolation, exclusively to the neuroectoderm of stage 11 embryos. Labeled cells were then dissociated and separated using magnetic cell sorting techniques, which permitted a high rate of purification of viable cells from the neuroectoderm as assayed by both cellular and molecular methods. Given the versatility and precision of the *GAL4-UAS* system and the increasing number of specific GAL4 lines, specific labeling of cell types or of tissue domains followed by cell sorting and microarray analysis should be possible for virtually all embryonic or postembryonic cell types and gene expression domains in *Drosophila*.

Improved identification of *gcm* downstream genes by microarray analysis of sorted neuroectodermal cells

Overexpression of *gcm* in the embryonic neuroectoderm of *Drosophila* followed by microarray analysis of differential gene expression based on whole mount embryos have been reported by two groups, including our group. In these studies, differential regulation of 417 potential *gcm* downstream genes (Egger et al., 2002) and 153 potential *gcm* downstream genes (Freeman et al., 2003) resulted from the microarray analyses. The whole mount-based microarray analysis of *gcm* downstream genes carried out as a control in our investigation resulted in 242 genes judged as differentially expressed. These relatively large gene numbers are in marked contrast to the 76 genes that were identified as differentially expressed in our sorted cell-based microarray analysis. A direct comparison of sorted cell-based versus whole mount-based microarray analyses is possible for our data, since both experiments were performed under virtually identical conditions (identical fly lines and developmental stages, genetic background, RNA isolation, microarray hybridization, microarray composition, and data analysis).

Based on the results, we strongly suggest that much more homogeneous nature of the target tissue obtained by cell sorting versus whole embryos lead to the fact that much fewer genes were judged to be differentially regulated in the sorted cell-based microarray experiment. This is because the GAL4-UAS method used limits *gcm* overexpression to precisely the same tissue that is labeled for subsequent magnetic cell sorting. Thus, signal loss due to “dilution” of neuroectodermal RNA by RNA obtained from other parts of the embryo is avoided, and tissue contamination from non-neuroectodermal cells that might be non-specifically affected by *gcm* action is minimized. However, we can not entirely rule out, that the smaller number of genes identified as differentially regulated is due to artifacts of the cell sorting or target amplification procedures used.

Independent confirmation of the improved gene identification rate obtained by microarray analysis from sorted cells is demonstrated by the results of in situ-based validation studies. Thus, among a group of 17 genes selected exclusively on their level of expression change in the microarray experiment, 9 had already been validated as *gcm* downstream genes by previous in situ hybridization studies, and a remaining 5 were validated by in situ experiments in this report. This implies a validation rate of 82%. Interestingly, an even higher validation

rate (91%) was obtained for the 13 genes that were judged as differentially expressed in both the cell sorted-based and the whole mount-based experiment. Taken together, these findings suggest that identification of *gcm*-downstream genes is markedly improved by sorted cell-based microarray analysis. However, more extensive and complete validation studies must be carried out on a gene-by-gene basis, before a full quantitative appreciation of the advantages of cell sorting for *gcm* target gene identification can be obtained.

GCM clearly regulates its target genes in a time and spatial restricted manner (Freeman et al., 2003). We selected a restricted developmental period for transcript profiling and it is therefore unlikely that our study identifies all the genes that act downstream of *gcm* to induce glial cell fate. Among other known GCM target genes our analysis failed to detect for example an upregulation for *pointed P1* (*pntP1*), *locomotion defects* (*loco*) and *tramtrack p69* (*ttk p69*). These genes might be induced at later stages by GCM and so act during glial differentiation (Granderath and Klambt, 1999). Despite the improved gene identification that is obtained by basing microarray analysis on homogeneous cell populations, it is clear that false positive results can not be entirely eliminated from microarray investigations. For example, hybridization errors will occur due to imperfections in the manufacture of microarrays. Moreover, the design of probe sets on the arrays that precisely represent all the genes in the genome yet eliminate cross-hybridization is difficult. Furthermore, annotation of a fully sequenced genome, such as that of *Drosophila*, remains tentative and indeed can change significantly during a period of improvement and validation (Misra et al., 2002), and this affects the ability of the microarray probe sets used on the arrays to detect the biologically correct gene. Finally, while the cell sorting method used was highly efficient in isolating cells and also gave excellent results in subsequent microarray analyses, some isolation-based artifacts can not be entirely ruled out. Since mechanical homogenization and enzymatic digestion were used to dissociate cells, it is possible that the transcriptome of the sorted cells may not be identical to that of the same cells in situ. For example, cleavage of surface proteins during dissociation might interfere with cell signaling and result in gene expression changes. Although this concern has been controlled here by monitoring gene expression of 3 genes, change of the global transcriptome can not be excluded. Moreover, given the relatively small numbers of cells obtained by sorting (as compared to the large cell numbers obtained by using entire embryos), PCR-based RNA amplification of targets for hybridization was necessary. Although the PCR-based RNA amplification method used has been reported to be sensitive

and lead to highly reproducible results (Ji et al., 2000), artifacts due to amplification also might influence in the results.

In summary, we have succeeded in applying MACS to isolate neuroectodermal cells from stage 11 embryos of *Drosophila* for microarray experiment which give much less false positive results than those based on whole mount tissue. Given that MACS only requires very simple and economic experimental settings and the specificity of the *GAL4/UAS* system, it will definitely facilitate the application of microarray techniques in *Drosophila*. In the meanwhile, the viable and relatively pure cells obtained via MACS provide important materials for experiments depending on access to specific cell types.

Acknowledgements:

We thank Antonius Rolink and his group (especially, Ulf Grawunder, Eva Harfst and Gina Balciunaite) for helping us with the FACSCalibur analysis, Markus Affolter, Carlos Ribeiro and Alain Jung for the initial idea of MACS in *Drosophila*, Sandra Klur for her generous help regarding the PCR-based microarray target preparation; Urs Kloter, Leandro Hermida, Philippe Demougin, Micheal Primig for technical support and helpful discussions. We specially thank Bruno Bello and Martin Müller for critical comments on the manuscript. This work was supported by the Swiss NSF.

TABLES

Table 1. Classification of differentially expressed genes according to molecular function

Molecular function(s)	Number of transcripts	
	sorted	whole mount
function unknown	41	124
enzyme	15	65
kinase /phosphatase	3	6
Protein/ carbohydrate/ nucleotide binding	2	7
nucleic acid binding	12	18
transcription factor	11	14
signal transducer	1	11
transcriptional regulator	0	2
transporter	1	6
cell adhesion molecule	1	2
chaperone	0	2
structural molecule	3	4
antioxidant	0	1
Total	76	242

Table 2. Overlap of differentially regulated genes of sorted and whole mount experiment
Previous: this gene has been validated as a potential *gcm* target by in situ hybridization in the previous published paper. In this study: this gene has been validated as a potential *gcm* target by in situ hybridization in this study.

Symbol	Fold changes	validation
cg3132	10.2	in this study
cg12910	9.4	previously
cg5822	5.3	previously
cg6218	5.3	previously
repo	4.6	in this study
cg6560	4.3	in this study
gcm	3.5	previously
cg3408	-2.0	--
al	-2.2	in this study
cg14041	-2.2	--
cg17649	-2.3	in this study
BcDNA:GH03482	-2.5	not validated
Nerfin-1	-2.6	in this study

FIGURES

Figure 1. Labeling of the embryonic neuroectodermal cells and targeted misexpression of *gcm* leads to gain of glial cells at the expense of neuronal cells.
(A,B) Immunostaining with anti-mCD8 in late stage 11 wild type-like embryos (A) and *gcm* misexpression embryos (B), which were labeled genetically with a transmembrane protein consisting of murine CD8 fused with GFP (green). Immunostaining shows the extent of labeling of the neuroectoderm; ventral view, anterior towards the left. (C,D) In situ hybridization of *gcm* in stage10 embryos shows expression in wild type-like embryos (C) and in *gcm* misexpression embryos (D), lateral views, anterior towards the left. In the wild type-like embryos, small clusters of cells in the neuroectoderm of the VNC express *gcm*; in *gcm* misexpression embryos, all cells of the neuroectoderm express *gcm*. (E,F) Immunostaining with anti-REPO antibody (red) in wild type-like embryos (E) and in *gcm* misexpression embryos (F); laser confocal microscopy of stage 11 embryos, ventral views of the VNC, anterior towards the left. In the wild type-like embryos, single glial precursors (which also express *gcm*; not shown) in each hemisegment express the *repo* gene. In *gcm* misexpression compared to wild type-like embryos, many more of the neuronal and glial precursor cells are REPO positive.

Figure 2. Assessment of extent of cell dissociation and determination of cell survival rate before sorting.

(A) Dissociated cells under normal filter; (B,C) Staining of dissociated cells with Hoechst 33342 (B) and with Propidium iodide (C); Hoechst 33342 staining (blue) reveals a majority of single cells as well as a few small clusters of non-dissociated cells. Examination of dissociated cell preparations with propidium iodide staining (red) shows that only very few cells were stained with propidium iodide. (D) Quantification of propidium iodide staining with flow cytometry. X-axis is the forward scatter. Y-axis is the fluorescent signal of propidium iodide staining. Each dot represents one cell. Cells within the rectangle R1 are judged as viable based on the low levels of propidium iodide staining. The number of cells in the rectangle R1 divided by the total cells counted gives a survival rate of over 90%.

Figure 3. Analysis of the degree of cell purification obtained by the MACS procedure according to the GFP fluorescent signal.

(A,B) Fluorescence microscopy of cells in the flowthrough fraction viewed under normal filter (A) and GFP filter (B); as expected, only a few cells in the flowthrough fraction were GFP positive (green) as judged by fluorescence microscopy. (C,D) Fluorescence microscopy of cells in the sorted fraction viewed under normal filter (C) and GFP filter (D); in contrast to the flow though fraction, the majority of the cells are GFP positive (green). (E) Quantification of the degree of GFP labeling of cells in the sorted fraction by flow cytometry. X-axis represents the GFP signal; Y-axis is the number of cells counted. M1 indicates the region in which cells are judged GFP positive; this region is delimited towards low intensities by the values of autofluorescence observed for GFP negative cells. The number of cells in M1 divided by the number of all cells counted gives the percentage of purification.

Figure 4. Quantitative transcript profiling of differentially expressed genes following *gcm* misexpression in cell sorting-based experiments.

Genes differentially expressed in response to misexpression of *gcm* in the sorted experiment were grouped according to functional classes. Bars represent the fold changes in expression level of cells sorted from the wild type-like embryos versus those from the *gcm* misexpression embryos. Positive values indicate that the relative expression level of a gene is increased (upregulation) following *gcm* misexpression and negative values indicate a decrease (downregulation). Ranges of signal intensity are given for the *gcm* misexpression condition as follows: white bars, signal intensity < 100; gray bars, signal intensity from 100-1,000; black bars, signal intensity > 1,000. 17 genes selected for in situ validation are indicated in bold: *genes validated previously and **genes validated in this study.

Figure 4.

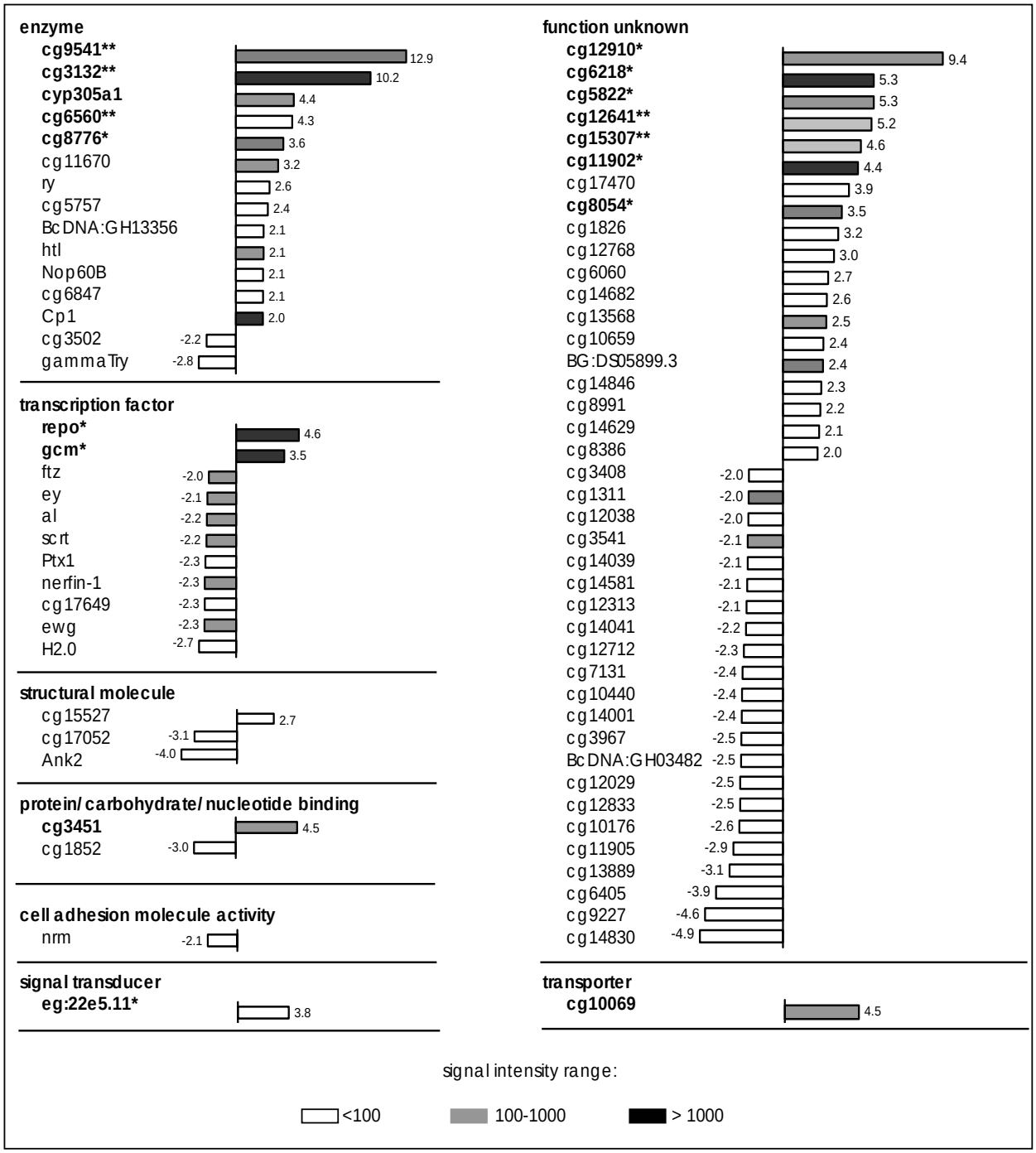
enzyme
cg9541** 12.9
cg3132** 10.2
cyp305a1 4.4
cg6560** 4.3
cg8776* 3.6
cg11670 3.2
ry 2.6
cg5757 2.4
BcDNA:GH13356 2.1
htl 2.1
Nop60B 2.1
cg6847 2.1
Cp1 2.0
cg3502 -2.2
gammaTry -2.8
transcription factor
repo* 4.6
gcm* 3.5
ftz -2.0
ey -2.1
al -2.2
scrt -2.2
Ptx1 -2.3
nerfin-1 -2.3
cg17649 -2.3
ewg -2.3
H2.0 -2.7
structural molecule
cg15527 2.7
cg17052 -3.1
Ank2 -4.0
protein/ carbohydrate/ nucleotide binding
cg3451 4.5
cg1852 -3.0
cell adhesion molecule activity
nrm -2.1
signal transducer
eg:22e5.11* 3.8
function unknown
cg12910* 9.4
cg6218* 5.3
cg5822* 5.3
cg12641** 5.2
cg15307** 4.6
cg11902* 4.4
cg17470 3.9
cg8054* 3.5
cg1826 3.2
cg12768 3.0
cg6060 2.7
cg14682 2.6
cg13568 2.5
cg10659 2.4
BG:DS05899.3 2.4
cg14846 2.3
cg8991 2.2
cg14629 2.1
cg8386 2.0
cg3408 -2.0
cg1311 -2.0
cg12038 -2.0
cg3541 -2.1
cg14039 -2.1
cg14581 -2.1
cg12313 -2.1
cg14041 -2.2
cg12712 -2.3
cg7131 -2.4
cg10440 -2.4
cg14001 -2.4
cg3967 -2.5
BcDNA:GH03482 -2.5
cg12029 -2.5
cg12833 -2.5
cg10176 -2.6
cg11905 -2.9
cg13889 -3.1
cg6405 -3.9
cg9227 -4.6
cg14830 -4.9
transporter
cg10069 4.5
signal intensity range:
<100
100-1000
> 1000

Figure 5. Spatial expression of selected candidate *gcm* downstream genes by in situ hybridization.

Whole-mount in situ hybridization shows expression of differentially regulated genes in wild type-like and *gcm* misexpression embryos. Lateral views (A-F) and ventral views (G-P) of stage 11 embryos, anterior is to the left. Differential expression fold changes and p values from corresponding microarray experiments are indicated on the right. (A,C,E) The *cg9541*, *cg15307* and *cg12641* genes are expressed broadly in the neuroectoderm of wild type-like embryos; (B,D,F) *gcm* misexpression in the neuroectoderm results in ectopic expression of *cg9541*, *cg15307* and *cg12641* in specific, segmentally repeated domains of the ventral neuroectoderm. (G,I) The expression domains of the genes *cg3132* and *cg6560* are restricted to specific groups of neuroectodermal cells in the wild type-like situation. (H,J) Following *gcm* misexpression embryos, increased expression of *cg3132* and *cg6560* results around these groups of neuroectodermal cells. (K, M) The genes *nerfin-1* and *cg17649* were expressed in a comparable set of cells in the neuroectoderm in the wild type-like situation. (L,N) Following *gcm* misexpression throughout the neuroectoderm, the expression of *cg13906* and *cg17649* was strongly reduced or even abolished in some of these cells. (O,P) In the wild type-like embryos, a segmentally repeated pattern of *al* expression is seen in three thoracic and eight abdominal lateral patches. Following *gcm* misexpression in the neuroectoderm, the expression pattern of *al* in the abdominal patches is repressed.

5. The *egghead* gene is required for compartmentalization in *Drosophila* optic lobe development

Yun Fan[1], Matthias Soller[2], Susanne Flister[1], Martin Hollmann[3], Martin Müller[1], Bruno Bello[1], Boris Egger[4], Kalpana White[2], Mireille A. Schäfer[3] and Heinrich Reichert[1*]

[1]Biozentrum/Pharmazentrum, University of Basel, CH-4056 Basel, Switzerland
[2]Department of Biology and Volen Center for Complex Systems, Brandeis University, Massachusetts 02454, USA
[3]FB18 Zoologie/Entwicklungsbiologie, University of Kassel, Kassel 34132, Germany
[4]Wellcome Trust/Cancer Research Gurdon Institute, University of Cambridge, Cambridge CB2 1QN, U.K.

Running Title: *egghead* in optic lobe development

Key Words: *egghead*, Compartment boundary, Photoreceptor axon targeting, Optic lobe, Glycosyltransferase, *Drosophila*

Summary

The correct targeting of photoreceptor neurons (R-cells) in the developing *Drosophila* visual system requires multiple guidance systems in the eye-brain complex as well as the precise organization of the target area. Here, we report that the *egghead* (*egh*) gene, encoding a glycosyltransferase, is required for a compartment boundary between lamina glia and lobula cortex, which is necessary for appropriate R1-R6 innervation of the lamina. In the absence of *egh*, R1-R6 axons form a disorganized lamina plexus and some R1-R6 axons project abnormally to the medulla instead of the lamina. Mosaic analysis demonstrates that this is not due to a loss of *egh* function in the eye or in the neurons and glia of the lamina. Rather, as indicated by clonal analysis and cell-specific genetic rescue experiments, *egh* is required in cells of the lobula complex primordium which transiently abuts the lamina and medulla in the developing larval brain. In the absence of *egh*, perturbation of sheath-like glial processes occurs at the boundary region delimiting lamina glia and lobula cortex, and inappropriate invasion of lobula cortex cells across this boundary region disrupts the pattern of lamina glia resulting in inappropriate R1-R6 innervation. Our genetic analysis thus uncovers a novel role of *egh* gene function in the developing *Drosophila* visual system and underscores the importance of the lamina/lobula compartment boundary in R1-R6 axon targeting.

Introduction

Compartment boundaries play key roles in pattern formation during development, and the establishment of these boundaries is thought to be a general mechanism for creating the organization of different tissues in a multi-cellular organism (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000). Multiple compartments have been identified in developing vertebrate and invertebrate central nervous systems (Lumsden and Krumlauf, 1996; Meinertzhagen and Hanson, 1993), and a number of molecules including cell-cell signaling proteins and transcription factors have been implicated in their establishment (Cheng et al., 2004; Cordes and Barsh, 1994; Schneider-Maunoury et al., 1997; Zeltser et al., 2001). During brain development, different cellular compartments form a complex prepatterned environment which is required for the navigation of axons to their correct targets. For example, in the developing mammalian brain, the subplate, the ganglionic eminence and the thalamic reticular complex are involved in the patterning of connections between the thalamus and the cortex (Ghosh and Shatz, 1993; Metin and Godement, 1996; Mitrofanis and Baker, 1993). Similarly, in the developing orthopteran brain, glial boundaries of compartment-like proliferative clusters are used by axons of pioneering neurons for the establishment of the primary axon scaffold that interconnects protocerebrum, deutocerebrum and tritocerebrum (Boyan et al., 1995; Reichert and Boyan, 1997).

The fly visual system is an excellent model system for the study of cellular and molecular mechanisms of axon guidance. The adult *Drosophila* compound eye comprises some 750 ommatidia, each containing eight photoreceptor neurons (R-cells). During larval development, different classes of R-cells in the eye disc project through the optic stalk to a different synaptic layer in the brain. R1-R6 axons terminate in the lamina between rows of epithelial and marginal glial cells, forming the lamina plexus, while R7 and R8 axons pass through the lamina and terminate in the medulla (Araujo and Tear, 2003; Clandinin and Zipursky, 2002, Meinertzhagen and Hanson, 1993; Tayler and Garrity, 2003). The formation of this R-cell projection pattern is known to involve complex bidirectional interactions between R-cell axons and different populations of cells in the target area. The molecular mechanisms that underlie these interactions have been studied intensively in the photoreceptor neurons of the developing eye, and to a lesser degree in the developing lamina. Thus, R-cells express a set of genes encoding cell surface receptors, signaling molecules and nuclear factors

that have been shown to control target selection in lamina and medulla (Cafferty et al., 2004; Clandinin et al., 2001; Garrity et al., 1996; Garrity et al., 1999; Kaminker et al., 2002; Lee et al., 2001; Lee et al., 2003; Maurel-Zaffran et al., 2001; Rao et al., 2000; Rawson et al., 2005; Ruan et al., 1999; Senti et al., 2000; Senti et al., 2003; Su et al., 2000; Yang and Kunes, 2004). In the lamina, glial cells appear to act as intermediate targets for R-cell axons and may be an important source of targeting information. When the organization of lamina glia is disrupted, large numbers of R1-R6 axons project through the lamina into the medulla (Poeck et al., 2001; Suh et al. 2002).

Unlike the lamina and medulla, the mature lobula complex, composed of lobula and lobula plate, does not receive direct input from R-cells in the adult fly brain. However, during optic lobe development, morphogenetic movements of the optic lobe anlagen transiently bring the lobula complex primordium into close apposition to the developing lamina and medulla (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993; Nassif et al., 2003). Given this spatial proximity, correct targeting of R-cell axons and plexus formation in the developing lamina can be influenced by cells of the lobula complex primordium, especially if the formation of the boundary that separates the developing lamina from the lobula complex is disrupted. Evidence for a perturbation of the R-cell projection pattern due to invasion of the developing lamina by cells of the adjacent lobula complex has been obtained in *slit* and *robo* loss-of-function mutants, in which the lamina/lobula cortex boundary is disrupted resulting in cell mixing across the two optic lobe compartments (Tayler et al., 2004).

In this report, we investigate the role of the *egghead* (*egh*) gene in the formation of the R-cell projection pattern. Our findings show that in *egh* loss-of-function mutants, R1-R6 axons form a disorganized projection pattern characterized by defects in the lamina plexus and aberrant projection of some R1-R6 axons through the lamina and into the medulla. Genetic analysis involving mosaics demonstrate that these defects are not due to a loss of *egh* function in the eye or in the neurons and glia of the lamina. Instead, clonal analysis and cell-specific genetic rescue experiments show that *egh* is required in the cells of the lobula complex primordium. In the absence of *egh*, the lamina/lobula cortex boundary is disrupted as indicated by the disorganization of sheath-like glial processes at the interface between lamina glia and distal cells of the lobula cortex. Cell mixing across the lamina/lobula cortex boundary occurs, and neurons of the lobula cortex invade the developing lamina at the site of lamina plexus formation disrupting the pattern of lamina glia and resulting in inappropriate R1-R6 axonal

projections. This finding uncovers a novel role of the *egh* gene in the developing *Drosophila* visual system and provides further support for the unexpected role of the lamina/lobula compartment boundary in R1-R6 axon targeting.

Materials and methods

Fly strains

All stocks were reared on standard cornmeal medium (Ashburner, 1989) at 25°C unless indicated otherwise. The alleles egh^7 and egh^3 were originally isolated as members of the zw^4 complementation group (Judd et al., 1972) and were further described by Goode et al (Goode et al., 1996). egh^7 resulted from a point mutation that changes the conserved Methionine at position 308 to Lysine and is biochemically a strong hypomorph (Wandall et al., 2005). egh^3 is associated with a 2.4kb deletion that is expected to remove information from the 3´ ends of both the *egh* and *KLP3A* genes (Williams et al., 1995). Both *egh* alleles were rebalanced with *FM7c Kr-GAL4 UAS-GFP* (Casso et al., 2000). *Ro-τlacZ*, *1.3D2-GAL4*, *gcm-GAL4* and *repo-GAL4* were kindly provided by I. Salecker. The following chromosomes were obtained from the Bloomington Stock Center. X chromosome: *FM7 actin-GFP*, *UAS-CD8::GFP, y w FRT19A*, GMR-*hid l(1)CL hsFLP FRT19A* and *tub-GAL80 hsFLP FRT19A*. Chromosome II: GMR-*GAL4*, *sca-GAL4*, *UAS-nlslacZ* and *UAS-CD8::GFP*. Chromosome III: *ey-FLP*, *ey-GAL4 UAS-FLP*, *tub-GAL4* and *C855a-GAL4*. The following strains were generated for rescue experiments: (1) egh^7/*FM7 actin-GFP*; *UAS-egh*. (2) egh^7 *UAS-CD8::GFP*/*FM7 actin-GFP*; *UAS-egh*.

Molecular biology and transformation

The *egh* gene extends over approximately 10kb and comprises 4 exons and 3 introns (detailed data are available in FlyBase). To generate *UAS-egh*, a PCR fragment containing 282bp preceding the ATG, the complete *egh* open reading frame as well as intron 3 was amplified and subcloned into the pUAST vector (Brand and Perrimon, 1993). Transgenic lines were generated according to standard procedures (Rubin and Sprading, 1982).

Mosaic Analysis

For the mosaic analysis in the adult eye, the Spurr-embedded eye sections were carried out on adult flies of the following genotype: y^+ *egh*^{7}*w FRT19A*/*tub-GAL80*[w^+] *hsFLP*[ry^+] *FRT19A*; *ey-FLP*[ry^+]/+. Homozygous y^+ *egh*^{7}*w FRT19A* clones were identified by the absence of red pigment granules due to loss of the miniwhite activity associated with *tub-GAL80*[w^+]. Experimental and control flies were aged for at least 7 days before sectioning to allow sufficient pigment accumulation. Only ommatidia with w^- pigment cells were analyzed. To examine the targeting of *egh* mutant R2-R5 axons in the wild type brain hemisphere during larval development, the late third instar larvae of the following genotype were analyzed: *egh*^{7} *FRT19A*/GMR-*hid l(1)CL FRT19A*; *ey-GAL4 UAS-FLP*/*Ro-τlacZ*. Larvae used as wild type control were: *FRT19A*/GMR-*hid l(1)CL FRT19A*; *ey-GAL4 UAS-FLP*/*Ro-τlacZ*. For the MARCM analysis in the optic lobe, 8-20hr posthatching larvae of the following genotypes were heat shocked for 1hr at 37°C, raised at 25°C, and analyzed at the late third instar larval stage: (1) Generation of *egh* mutant clones: *egh*^{7} *FRT19A*/*tub-GAL80 hsFLP FRT19A*; *UAS-nlslacZ UAS-CD8::GFP*/+; *tub-GAL4*/+. (2) Clonal rescue experiment: *egh*^{7} *FRT19A*/*tub-GAL80 hsFLP FRT19A*; *UAS-nlslacZ UAS-CD8::GFP*/*UAS-egh*; *tub-GAL4*/+.

Histology

Whole-mount immunochemistry labeling protocols were as described by Garrity et al (Garrity et al., 1996). The following primary antibodies were used: mAb24B10, mouse anti-β-Gal, mouse anti-Dac, mouse anti-ELAV, mouse anti-Repo, mouse anti-FasIII, mouse anti-Slit and mouse anti-Robo (all 1:50; Developmental Studies Hybridoma Bank), rabbit anti-Repo (1:500; Halter et al., 1995) and FITC-conjugated goat anti-HRP (1:20; Jackson Immunoresearch). For fluorescent labeling, secondary antibodies were Alexa488, Alexa568 and Alexa647 conjugated (all 1:150; Molecular Probes). For laser confocal microscopy, a Leica TCS SP was used. The Spurr-embedded sections of adult eyes were prepared as described by Basler and Hafen (Basler and Hafen, 1988). *egh* probes for RNA in situ hybridization (Tautz and Pfeifle, 1989) were obtained by using the coding sequence of *egh* gene as template. In situ hybridizations on the larval brain were carried out as described by Poeck et al (Poeck et al., 1993).

Results

The R-cell projection pattern is disrupted in *egghead* mutants

The *egh* gene is essential for embryonic epithelial development and oogenesis (Goode et al., 1996; Rubsam et al., 1998). Sequence analysis as well as enzymatic assays suggest that *egh* encodes a glycosyltransferase and functions in a glycosylation pathway (Wandall et al., 2003). Given that some of the features of *egh* action are reminiscent of neurogenic gene action (Goode et al., 1996), we investigated the role of *egh* in nervous system development. No obvious zygotic phenotypes of *egh* loss-of-function was observed in the embryonic nervous system, however, clear defects of R-cell axonal connectivity were seen by using the marker mAb24B10 (Zipursky et al., 1984) in third instar larval brains (Fig. 1A-C). Thus, in *egh* mutants, egh^{7} and egh^{3}, the lamina plexus was discontinuous and of variable thickness. In addition, thicker axon bundles were found projecting to the medulla in comparison to the wild type situation.

Further analysis of *egh* mutants using *Ro-τlacZ*, a marker selectively expressed in R2-R5 axons (Heberlein and Rubin, 1990), showed that some R2-R5 (and we infer R1-R6) axon fascicles fail to stop in the lamina and, instead, projected to the medulla. Moreover, the R2-R5 axons that still terminated in the lamina, were disorganized, showed perturbed fasciculation and formed abnormal lamina plexus patches when compared to wild type (Fig. 1D-F). The *egh* photoreceptor projection defects were fully rescued by placing a *UAS-egh* transgene under the control of the ubiquitously expressed *tub-GAL4* (Lee and Luo, 1999) driver in an *egh* mutant background (see below). These results indicate that *egh* is required for correct R1-R6 axonal projections during larval development.

egghead is not required in the eye for the formation of the correct R-cell projection pattern

To determine if *egh* is required in R-cells for correct axonal projections, an *eyeless*-FLP/FRT system was used to induce eye-specific mitotic recombination creating *egh* homozygous mutant ommatidia (Newsome et al., 2000). Adult flies that contain large *egh* homozygous mutant clones in the eye were then analyzed by histological sections. Compared to the wild

type, more than 98% *egh* mutant ommatidia (n = 190/193 examined in 7 eyes) had a normal number and array of R-cells (Fig. 2A). This suggests that R-cell fate determination and differentiation are largely normal in *egh* mutants.

Subsequently, the *Ro-τlacZ* marker was used in combination with an eye-specific mosaic technique (Stowers and Schwarz, 1999) to assess *egh* mutant R1-R6 axonal projections in wild type larval brains. Due to this mosaic technique, homozygous mutant clones cover over 90% of the photoreceptors in the late third instar larval eye disc (Soller and White, 2003). The labeled projections of predominantly *egh* mutant R-cells in the optic lobes were indistinguishable from the wild type control (Fig. 2B,C). These findings indicate that *egh* function is not required in R-cells and their axons for the formation of a normal R-cell projection pattern. This implies that *egh* is required in the general target area of these axons in the developing optic lobes.

The arrangement of lamina glia and the lamina/lobula cortex boundary are disrupted in *egghead* mutants

Lamina neurons are generated from a subpopulation of neuroblasts in the outer proliferation center (OPC) (Salecker et al., 1998). In a two-step process, neuroblasts give rise to lamina precursor cells (LPCs) and LPCs subsequently complete final divisions to produce mature lamina neurons. During this process, R-cell afferents release signals such as Hedgehog and Spitz to induce lamina neuron development. In turn, LPC progeny assemble into lamina columns which associate with older R-cell axon bundles. To assess lamina neuron differentiation in *egh* mutants, the early neuronal differentiation marker Dachshund (Dac, Mardon et al., 1994) and the late neuronal differentiation marker ELAV (Robinow et al., 1988) were used. The expression pattern of Dac in the lamina was indistinguishable in wild type and *egh* mutants (Fig. 3A,B). Moreover, as in wild type, mature lamina neurons L1-L5, which form lamina columns, expressed ELAV in *egh* mutants (Fig. 3C,D). In *egh* mutants and in wild type, L1-L4 neurons formed a superficial layer, while L5 neurons resided in a medial layer which was just above the epithelial glia cells (Huang and Kunes, 1998). These findings imply that *egh* is not required for the generation and differentiation of lamina neurons.

Lamina glial cells are generated by glial precursor cells located in two domains at the dorsal and ventral edges of the prospective lamina (Huang and Kunes, 1998). Mature glia migrate

into the lamina target field along scaffold axons which serve as migratory guides (Dearborn and Kunes, 2004). Lamina glial cells have been identified as the intermediate targets of R1-R6 axons, and removal of glia disrupts R1-R6 axon targeting (Poeck et al., 2001). In wild type, R1-R6 growth cones terminated between rows of epithelial and marginal glial cells, and the row of medulla glial cells lay beneath the marginal glial cells (Fig. 4A). In *egh* mutants, a layered assembly of glial cells was also found at the site of lamina plexus formation, however, these layers were clearly disorganized as compared to the wild type situation (Fig. 4B). Notably, defects in glial layer organization correlated with the gaps in the associated lamina plexus. This suggests that *egh* is not required for the initial generation and migration of glial cells into the target area, but that the final pattern of glial cells in the developing lamina is perturbed in *egh* mutants.

Cells of the lobula complex are derived from the inner proliferation center (IPC). During optic lobe development, the lobula complex primordium transiently moves into close apposition to the developing lamina (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993; Nassif et al., 2003). Recently, the existence of a boundary region between the developing lamina and lobula cortex has been demonstrated, and evidence for a perturbation of the R-cell projection pattern due to the invasion of the developing lamina by cells of the lobula cortex has been obtained in *slit* and *robo* loss-of-function experiments (Tayler et al., 2004). A comparable phenotype was observed in *egh* mutants. Thus, in wild type, lobula distal cell neurons, which form the anterior edge of the lobula cortex, were separated from the adjacent posterior face of the developing lamina by a precise boundary region (Fig. 4C,E). In contrast, in *egh* mutants, this boundary region between lobula and lamina was no longer apparent, and streams of lobula distal cell neurons crossed into the base of the developing lamina (Fig. 4D,F). Moreover, these sites of lobula distal cell neuron invasion correlated with sites of structural defects in the developing lamina plexus.

egghead is required in cells of the lobula complex primordium for the formation of the correct R-cell projection pattern

In situ hybridization experiments performed in the third instar larval brain showed that *egh* is expressed broadly in the developing optic lobes as well as in the central brain and ventral nerve cord (data not shown). In order to determine in which cells *egh* is required for the

formation of a correct R-cell projection pattern, a series of clonal analyses were carried out by using MARCM (Lee and Luo, 1999).

Lamina neurons were examined first. Although the early differentiation of lamina neurons appears normal (see above), it is possible that *egh* is required in these cells for correct R1-R6 axonal projections. However, in all mosaic animals (n = 28) with *egh* mutant clones in lamina precursors and lamina neurons, the R-cell projection pattern appeared normal (Fig. 5A-C). This suggests that *egh* is not required in lamina neurons for correct R-cell axonal projections.

To determine if *egh* is required in the glial cells of the target area for the formation of the correct R-cell projection pattern, we used MARCM to generate labeled clones of epithelial and marginal glial cells. Large *egh* homozygous mutant clones containing glial precursor cells as well as epithelial and marginal glial cells were examined in the optic lobe. In all animals (n = 26) with such large clones, labeled *egh* mutant glial cells were always arranged in layers at the site of the developing lamina plexus and these layers did not show obvious defects in their organization. Moreover, loss of *egh* in these cells did not result in R-cell projection defects (Fig. 5D-F). This suggests that *egh* is not required in epithelial and marginal glial cells for the formation of the correct R-cell projection pattern.

In contrast, *egh* mutant MARCM clones in the lobula complex primordium were usually (74%, n = 27) associated with specific defects in the lamina plexus (Fig. 5G-I). These defects were visible as gaps or perturbations in the lamina plexus that were consistently located in the region where the mutant lobula cell clones contact the lamina plexus. This type of projection defect could be rescued by expression of an *egh* tansgene in the MARCM $egh^{-/-}$ clone (data not shown). These clonal analyses suggest that *egh* is required in the lobula complex primordium for correct R-cell axonal projections.

Although consistent in occurrence, the axonal projection defects observed in MARCM *egh* mutant clones were smaller than those seen in whole *egh* mutant animals. This may be due to the restricted size of the MARCM mutant clones that are generated in the optic lobe. To provide further evidence for a requirement of *egh* in the lobula complex primordium, genetic rescue experiments were carried out. In these experiments, performed in an *egh* mutant background, the expression of *UAS-egh* was placed under the control of different GAL4 drivers (Table 1). Ubiquitous expression of *UAS-egh* via *tub-GAL4* fully rescued the

projection phenotype in *egh* mutants. In contrast, the eye-specific driver *GMR-GAL4* (Freeman, 1996) and the glial-specific driver *1.3D2-GAL4* (Garrity et al., 1999) did not rescue the projection phenotype. Similarly, *gcm-GAL4*, which in the third instar larval brain drives expression in LPCs, lamina neurons, epithelial and marginal glia (Iris Salecker, personal communication; Ting et al., 2005), did not rescue the projection defect. However, the *C855a-GAL4* driver (Manseau et al., 1997) did rescue the *egh* projection phenotype in more than 80% of the brain hemispheres inspected (Fig. 5J,K). In the third instar larval brain, *C855a-GAL4* drives expression throughout most of the lobula complex primordium and the IPC, as well as in cells of the developing lamina including LPCs (Fig. 5L). Since expression of *egh* in the developing eye and the cells of the developing lamina alone did not rescue the defect, this cell-specific rescue experiment provides further support for the notion that *egh* is required in the lobula complex primordium for correct targeting of R1-R6 axons in the developing lamina.

Glial processes at the lamina/lobula cortex boundary are perturbed in *egghead* mutants

Given that lobula distal cell invasion of the developing lamina in *egh* mutants correlates with the absence of a precise lamina/lobula cortex boundary region, this region was analyzed in more detail. The boundary between the developing lamina and lobula complex primordium was delimited by sheath-like glial processes that extend from the satellite glia near the lateral surface of the brain to the posterior face of the lamina plexus (Fig. 6A,A'; arrowheads). At the posterior edge of the lamina, these satellite glial processes interfaced with processes from epithelial, marginal and medulla glial cells. Sheath-like glial processes of this type are thought to establish and stabilize compartments in the developing larval brain (Younossi-Hartenstein et al., 2003; Pereanu et al., 2005). In *egh* mutants, these sheath-like glial processes were severely disrupted or missing altogether and the arrangement of the satellite and lamina glia was disorganized (Fig. 6 B,B'). Notably, the perturbed arrangement of the lamina glial cells correlated spatially with the site of invasion of distal cell neurons into the lamina and the extensive intermingling of the invading cells with the lamina glia (compare Fig. 4E,F). This is consistent with a role of glia at the lamina/lobula cortex boundary region in preventing cell mixing between compartments.

The similarity of the cell invasion phenotype seen in *egh* mutants and in *slit* and *robo* loss-of-function experiments (Tayler et al., 2004) prompted analysis of the expression of Slit and

Robo in *egh* mutants. The expression of Slit in the developing optic lobes of *egh* mutants appeared normal. In mutants, as in wild type, Slit expression was observed surrounding the lamina glial cells and reached highest levels in the medulla neuropile (data not shown). Similarly, the expression of Robo in the developing optic lobes of *egh* mutants appeared largely normal. Thus, in *egh* mutants as in the wild type, Robo was expressed broadly in the optic lobe including medulla and lobula cortex and was also seen delimiting the anterior and posterior edges of the lamina glia (Fig. 6C,D). Although a slight decrease of Robo expression at the border of the lamina glia/lobula cortex in *egh* mutants versus to wild type was observed (Fig 6C',D', arrow), this is probably due to the fact that cells of the lobula cortex have Robo on their surface and that these cells are mislocated in the *egh* mutant.

Discussion

Our genetic analysis indicates that the *egh* gene is required in visual system development. In the absence of *egh*, a number of mutant phenotypes occur in the optic lobe: (1) disruption of R1-R6 axon targeting in the lamina, (2) perturbation of lamina glial organization, (3) invasion of lobula cortex distal cells into the lamina, and (4) disruption of the glial sheath at the lamina/lobula cortex boundary region. A simplified summary scheme of this is shown in figure 7. We hypothesize that these phenotypes are causally related in *egh* mutants, in that a disruption of the lamina/lobula boundary allows lobula cortex distal cells to invade the adjacent lamina and displace lamina glial cells, resulting in aberrant photoreceptor projection patterns. In the following we discuss the evidence for and against this hypothesis.

The disruption of R1-R6 targeting is due to misarrangement of lamina glia

The generation of the R-cell projection pattern involves complex bidirectional interactions between R-cell axons and different populations of cells in the target region (Chotard and Salecker, 2004; Tayler and Garrity, 2003). R-cell axons provide signals for induction of proliferation and differentiation of lamina neurons and for differentiation and migration of glial cells. In turn, lamina glial cells act as intermediate targets for R1-R6 growth cones. When these glial cells are missing or reduced, as occurs in *nonstop* and *jab1/csn5* mutants,

large numbers of R1-R6 axons project aberrantly through the lamina into the medulla. Given this crucial role of lamina glia for correct R1-R6 axonal projections, the disorganization of lamina glia in *egh* mutants is likely to result in aberrant R1-R6 projection patterns. Indeed, in *egh* mutants, defects in lamina glial layer organization correlate spatially with defects in the associated lamina plexus.

It is conceivable that the aberrant R-cell projection in *egh* mutants might be due, at least in part, to defects in lamina neurons, which are the final targets of R1-R6 axons. However, in *egh* mutants, generation and differentiation of lamina neurons appear normal, and animals with MARCM mutant clones in lamina neurons have normal R-cell projection patterns. Defects in R-cells themselves also unlikely contribute substantially to the projection defect since *egh* mutant R1-R6 photoreceptors project normally into wild type optic lobes, and R-cell fate and determination appear normal in *egh* mutant clones in the eye. Thus, the most reasonable explanation for the disrupted R-cell axonal projections in *egh* mutants is that they are a consequence of the perturbation of lamina glia.

The misarrangement of lamina glia is due to invasion of lobula distal cells

Lamina glia cells migrate to the lamina target field from their progenitor zones (Huang and Kunes, 1998). In *egh* mutants, the initial generation and migration of glial cells to the lamina appears unaffected. Epithelial and marginal glia in large *egh* mutant clones which contain the glia and their precursors, are arranged normally in appropriate layers at the site of formation of a normal lamina plexus. Moreover, expression of Egh protein in the lamina glia in *egh* mutants does not rescue the phenotype. It, therefore, seems unlikely that the mispositioning of glial cells in *egh* mutants is due to defects in the glial cells, their precursors or their migratory behavior.

These observations imply that the mispositioning of lamina glia in *egh* mutants is a secondary consequence of other disruptions in optic lobe development. MARCM mutant clonal analysis indicates that the characteristic defects in the lamina plexus are associated with cells of the lobula complex primordium. Moreover, the *egh* mutant phenotype is rescued in experiments in which Egh protein is expressed in the lobula cortex. A good candidate for the lobula-associated disruption in optic lobe development in *egh* mutants is the observed invasion of lobula cells into the base of the developing lamina. In *egh* mutants, the distal cell neurons

invade and intermingle with lamina glial cells, and this cell intermixing correlates spatially with the displacement of the lamina glia at the base of the developing lamina. We can not rule out the possibility that the displacement of the lamina glia is caused primarily by unidentified signals from the lobula cortex, with distal cell invasion into the disrupted glial layers occurring secondarily. However, the most reasonable explanation for the observed glial cell mispositioning phenotype is that it is due to the invasion and intermingling of lobula cells into the lamina.

The invasion of lobula distal cells is due to defects at the lamina/lobula cortex boundary

Glial cells are thought to play a major role in the formation and maintenance of many compartments in the central nervous system, and some of the most prominent compartments in the insect brain, including the optic ganglia, are delimited by sheath-like glial septa (Hahnlein and Bicker, 1996; Boyan et al., 1995; Younossi-Hartenstein et al., 2003; Pereanu et al., 2005). In the developing visual system of *Drosophila*, the cells of the developing lamina which derive from the OPC are transiently adjacent to the cells of the developing lobula complex which derive from the IPC. In the wild type, no intermixing of the two cell populations occurs, and the boundary area that separates these two compartments is delimited by sheath-like glial cell processes which extend from the lateral surface of the brain to the posterior face of the developing lamina plexus. This boundary region is also the site of molecular interactions between the Slit and Robo family proteins (Tayler et al., 2004).

Both the glial sheath and the Slit/Robo interface are likely to be involved in compartmentalization and prevent invasion and cell intermingling across the lamina/lobula cortex boundary. Evidence for a role of the Slit/Robo interface in boundary maintenance comes from loss-of-function studies; in the absence of functional Slit or Robo family proteins, lobula distal cell neurons invade the lamina, resulting in cell mixing across the lamina/lobula cortex boundary (Tayler et al., 2004). Evidence for a role of the sheath-like glial processes in boundary maintenance comes from *egh* loss-of-function mutants. In the absence of *egh*, these glial processes are severely disrupted, and this is invariably accompanied by the invasion of lobula cells into the lamina, again resulting in cell mixing across the lamina/lobula cortex boundary. How the *egh* gene contributes to the formation of the glial sheath interface at the lamina/lobula boundary is currently unknown.

Towards a molecular analysis of Egh function in compartmentalization

The genetic demonstration of an *egh* requirement in the developing optic lobe provides an entry point for a more detailed molecular analysis of compartment boundary formation or maintenance. How might the *egh* gene contribute to compartmentalization? Analysis of the Egh protein domains and structural motifs suggests that *egh* encodes a Golgi/ER-localized glycosyltransferase (Wandall et al., 2003). In vitro glycosyltransferase assays and in vivo analysis show that *egh* is capable of forming the ceramide core, mactosylceramide, and is essential for glycospingolipid biosynthesis (Wandall, et al., 2005). This, in turn, suggests that *egh* might be involved in regulating the organization of lipid composition in the plasma membrane by controlling the biosynthesis of glycospingolipids. In vertebrates, glycosphingolipids are known to have functions in cell adhesion, growth, regulation, differentiation, cell interaction, recognition and signaling (Watts, 2003), and all of these processes may contribute to compartment formation. Despite differences in the chemical structure of their lipids, *Drosophila* membranes contain microdomains with a similar protein and lipid composition as their mammalian counterparts which are believed to provide suitable microenvironments to enable selective protein-protein interactions as well as local initiation of signal transduction (Rietveld et al. 1999; Simons and Toomre, 2000; Tsui-Pierchala et al., 2002).

Interestingly, recent studies on heparan sulfate proteoglycans (HSPGs) suggest that a transmembrane HSPG, Syndecan (Sdc), and a glypican, Dally-like protein (Dlp), are involved in visual system assembly (Rawson et al., 2005). Both Sdc and Dlp are expressed in the lobula cortex, and during embryonic axonogenesis, Sdc is required for proper Slit/Robo signaling (Steigemann et al., 2004; Johnson et al., 2004). During visual system development, Dlp is required in both the retina and the optic lobes. Moreover, Dlp belongs to the glycosylphosphatidylinositol (GPI)-linked HSPGs and several studies suggest that particularly ordered lipid environments are enriched with certain membrane proteins involving GPI-anchored proteins (Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). In view of these findings, it will be important to investigate the possible roles of HPSGs, such as Sdc and Dlp, in mediating *egh* action on compartmentalization of *Drosophila* visual system.

Studies on oogenesis indicate that *egh* together with *brainiac*, another gene encoding a glycosyltransferase, are involved in *Notch*-mediated epithelial development and also interact with EGFR signaling pathway (Goode et al., 1992; Goode et al., 1996; Wandall et al., 2005). Since a role of Notch and EGFR signaling in compartmentalization has been reported (Micchelli and Blair, 1999; Rauskolb et al., 1999; Zecca and Struhl, 2002), further insight into these molecular pathways in *egh* mutants might help to understand the molecular mechanisms that mediate *egh* action in compartment boundaries.

Acknowledgments

We thank Alois Hofbauer, Karl-Friedrich Fischbach and Volker Hartenstein for helpful discussions. Franz Wittwer, Christoph Hugentobler and Ernst Hafen for help with spur-embedded thin sections and Ursula Sauder (Biozentrum Microscopy Center) for technical assistance. Special thanks go to Iris Salecker for fly lines as well as many comments and suggestions at various stages of this work. We are grateful to Gerd Technau, Joachim Urban, Iris Salecker, the Developmental Studies Hybridoma Bank and the Bloomington Stock Center for antibodies and flies. This work was supported by the SNSF.

TABLE

Table 1. Phenotypic Rescue of *egh*^{7} by cell specific GAL4 drivers

Drivers	GAL4 Expression in the Eye-brain Complex				Numbers of Hemispheres Tested	Number (%) Wild Type
	R-cells	LPCs, ln	eg, mg	lob		
None (*w*^{1118})	-	-	-	-	58	0
tub-GAL4	+	+	+	+	56	56 (100%)
GMR-*GAL4*	+	-	-	-	60	0
1.3D2-GAL4	-	-	+	-	52	0
gcm-GAL4	-	+	+	-	56	0
C855a-GAL4	-	+	+	+	66	55 (83.3%)

R-cells, photoreceptor neurons; LPCs, lamina precursor cells; ln, lamina neurons; eg, epithelial glia; mg, marginal glia; lob, lobula complex primordium.

FIGURES

Figure 1. R1-R6 axon targeting is disrupted in *egghead* mutants.

Laser confocal microscopy of late third instar visual systems, frontal views. (A-C) R-cell axons in wild type and *egh* mutants immunolabeled with mAb24B10. (A) In wild type, R1-R6 axons terminate in the lamina and form the lamina plexus. R7 and R8 axons project through the lamina and terminate in the medulla. (B,C) In *egh* mutants, egh^{7} and egh^{3}, the lamina plexus is separated by gaps (arrowheads). In the medulla, thicker axon bundles (arrows) are found compared to wild type. (D-F) R2-R5 axons in wild type and *egh* mutants visualized with *Ro-τlacZ*. (D) In wild type, labeled axons terminate in the lamina and form a well-organized part of the lamina plexus. (E,F) In egh^{7} and egh^{3} mutants, some labeled axons fail to terminate in the lamina and project into the medulla (arrows); axons that do terminate in the lamina are disorganized and form abnormal patches of the lamina plexus (arrowheads). ed, eye disc; os, optic stalk; la, lamina plexus; me, medulla; asterisk, larval optic neuropile. Scale bar, 20 μm.

Figure 2. *egghead* is not required in the eye for R-cell development and axon targeting.
(A) Adult eye, thin section. egh^{7} mutant ommatidia can be identified by the absence of red pigment granules (arrows). As in wild type, *egh* homozygous mutant ommatidia have a normal number and array of R-cells, and a normal size and shape of the rhabdomeres (arrowheads). (B,C) Laser confocal microscopy, frontal views of late third instar visual systems, R2-R5 axons visualized with *Ro-τlacZ*. (B) In wild type, labeled axons terminate in the lamina and form a well-organized lamina plexus. (C) In an eye-specific mosaic in which large sections of R-cells in the eye disc are egh^{7} mutant, the mutant R2-R5 axons also terminate in the lamina and form a well-organized lamina plexus. la, lamina plexus. Scale bars, 10μm (A), 20 μm (B,C).

Figure 3. Lamina neurons develop normally in *egghead* mutants.

Laser confocal microscopy of triple immunolabeled wild type (A,C) and egh^{7} mutant (B,D) late third instar visual systems. (A,B) Frontal views, (C,D) horizontal views. In wild type (A) and in *egh* mutants (B), expression of the early neuronal differentiation marker Dac (red) is normal and extends from the LPC region (arrows) throughout the lamina neuron region. Nevertheless, in the *egh* mutants, the lamina plexus is perturbed and separated by gaps (arrowheads). Lamina glia and satellite glia labeled with anti-Repo (blue), R-cell axons labeled by anti-HRP (green). In wild type (C) and in *egh* mutants (D), the late neuronal differentiation marker ELAV (red) is expressed in all mature lamina neurons (L1-L5). L1-L4 neurons contact each other and form a superficial layer, while L5 neurons reside in a medial layer which is just above the epithelial glia cells. Lamina glia and satellite glia labeled with anti-Repo (blue), R-cell axons labeled with mAb24B10 (green). gl, lamina glia; sg, satellite glia; la, lamina plexus; ln, lamina neurons. Scale bar, 20 μm.

Figure 4. The arrangement of lamina glia and the lamina/lobula cortex boundary are disrupted in *egghead* mutants.

Laser confocal microscopy of double immunolabeled wild type (A,C,E) and egh^{7} mutant (B,D,F) late third instar visual systems. (A,B) Frontal views, (C,D) lateral views, (E,F) horizontal views. (A,B) R-cell axons labeled with mAb24B10 (green), mature glial cells labeled with anti-Repo (red). (C,D) Medulla cortex and portions of the lobula cortex labeled with CD8::GFP driven by *sca-GAL4* (sca:GFP, green), R-cell axons labeled with mAb24B10 (red). (E,F) R-cell axons labeled with CD8::GFP driven by GMR-*GAL4* (GMR:GFP, green), IPC neuroblasts and distal cell neurons labeled with anti-FasIII (red). (A) In wild type, R1-R6 growth cones terminate between epithelial and marginal glia and form the lamina plexus. A third row of glial cells, the medulla glia, lies beneath the marginal glia. Satellite glia are interspersed among the lamina neurons. (B) In *egh* mutants, glial cells are present in the target area, but the three-layered pattern of glial cells in the developing lamina is perturbed, and the regions of photoreceptor axon mistargeting correlate with areas of lamina glial disruption (arrowheads). (C) In wild type, IPC neuroblasts and their distal cell neuron progeny are adjacent to the posterior edge of the lamina. (D) In *egh* mutants, distal cell neurons enter the posterior face of the lamina (arrows). (E) In wild type, distal cell neurons are immediately adjacent to the posterior face of the lamina (arrowhead). (F) In *egh* mutants, distal cell neurons cross into the base of the lamina (arrow) and reach the anterior part of the lamina (arrowhead). sg, satellite glia; eg, epithelial glia; meg, medulla glia; la, lamina plexus; medc, medulla cortex; dcn, distal cell neurons; IPC, inner proliferation center; medn, medulla neuropile. Scale bars, 20μm (A-D), 10 μm (E,F).

Figure 5. *egghead* is required in cells of the lobula complex primordium for normal R-cell axonal projections.

Laser confocal microscopy of late third instar visual systems, frontal views. (A-I) Larval brains with *egh* mutant MARCM clones. Mutant cells labeled with membrane-associated CD8::GFP (GFP, green), R-cell axons labeled with mAb24B10 (red), glial cells labeled with anti-Repo (blue). (J,K) R-cell axons labeled with mAb24B10 (red) in *egh* mutants with *C855a-GAL4* and in *egh* mutants with *C855a-GAL4*/*UAS-egh*. (L) R-cell axons labeled with mAb24B10 (red) and GFP-labeled cells (green) in *C855a-GAL4*/*UAS-GFP* line. (A-C) *egh* homozygous mutant clone in lamina neurons. The same single optical section of the eye-brain complex is double labeled with GFP and mAb24B10 (A) and single labeled with mAb24B10 (B). (C) Stack of merged sections including this single section. Neuroblasts adjacent to the developing lamina give rise to LPCs (arrows) and lamina neurons. Loss of *egh* in lamina neurons does not affect normal R-cell axonal projections. (D-F) *egh* homozygous mutant clone in epithelial and marginal glial cells. The same single optical section of the eye-brain complex is double labeled with GFP and mAb24B10 (D) and single labeled with mAb24B10 (E). (F) Stack of merged sections including this single section. Glial precursor cells (arrows) are generated in two domains at the dorsal and ventral edges of the prospective lamina and give rise to mature glial cells which migrate into the lamina target field. Epithelial and marginal glia are above or below the lamina plexus. Loss of *egh* in epithelial and marginal glia, the R-cell projection pattern is normal. (A MARCM clone for satellite glia and two other clones near the surface of the brain hemisphere (asterisks) are also visible.) (G-I) *egh* homozygous mutant clone in cells of the lobula complex primordium. The same stack of four 1 μm thick optical sections of the eye-brain complex is double labeled with GFP and mAb24B10 (G) and single labeled with mAb24B10 (H). (I) Stack of merged sections including these four. The lobula complex primordium is generated by the IPC and extends radially to the lateral surface of the brain hemisphere. In most animals (74%, n = 27) with comparably large *egh* mutant clones in the lobula complex primordium, the lamina plexus is disrupted, and disorganized parts of the lamina plexus are consistently located in the region where these $egh^{-/-}$ cells are in close contact with the lamina plexus (arrowheads). (J) The *C855a-GAL4* driver alone is unable to rescue the R-cell projection defects in *egh* mutants. (K) Expression of *UAS-egh* driven by *C855a-GAL4* rescues the R-cell projection phenotype in *egh* mutants. (L) Stack of five 1 μm thick optical sections of the eye-brain complex. The *C855a-GAL4* drives expression, as indicated by GFP markers, throughout most cells of the lobula complex primordium derived from the IPC, as well as in cells of the developing lamina including LPCs (arrows). ln, lamina neurons; la, lamina plexus; sg, satellite glia; eg, epithelial glia; mg, marginal glia; meg, medulla glia; lob, lobula complex primordium; IPC, inner proliferation center. Scale bars, 20 μm.

Figure 6. The boundary between lamina and lobula complex primordium is disrupted in *egghead* mutants.

Laser confocal microscopy of double/triple immunolabeled wild type (A,A′,C,C′) and egh^{7} mutant (B,B′,D,D′) late third instar visual systems, horizontal views. (A,B) Glial membranes labeled with CD8::GFP driven by *repo-GAL4* (repo:GFP, green), glial cell nuclei labeled with anti-Repo (blue), cells of the lobula complex primordium labeled with anti-FasIII (red). (A′,B′) Glial membranes labeled with CD8::GFP driven by *repo-GAL4* (green), and (A′,B′) are corresponding views of (A,B). (C,C′,D,D′) Glial membranes labeled with CD8::GFP driven by *repo-GAL4* (repo:GFP, green), Robo expression visualized with anti-Robo (red). (C′,D′) are high magnification views of the corresponding outlined regions in (C,D). (A,A′) In wild type, sheath-like glial processes from satellite glia and lamina glia delimit the lamina/lobula boundary (arrowheads), and lobula distal cell neurons are located adjacent to the posterior edge of the lamina glia. (B,B′) In *egh* mutants, the organization of the sheath-like glial processes at the lamina/lobula boundary (arrowheads) is severely perturbed and the arrangement of the satellite and lamina glia is disrupted. (C,C′) In wild type, Robo is highly expressed in the medulla neuropile as well as at the anterior and posterior (arrow) face of the lamina glia. (D,D′) In *egh* mutants, Robo expression remains largely normal although Robo expression at the posterior lamina glia/lobula cortex boundary appears slightly reduced (arrow). sg, satellite glia; gl, lamina glia; lob, lobula complex primordium; medn, medulla neuropile. Scale bars, 10 μm (A, B, C, D).

Figure 7. A schematic summary diagram of visual system development in wild type and *egh* mutants.

Horizontal views. In wild type, precise boundary regions separate the developing lamina, lobula cortex and medulla cortex. In *egh* mutants, the boundary between lamina and lobula cortex is disrupted, cells of lobula complex invade the lamina, and displace the lamina glia, thus, resulting in disturbed R1-R6 axon targeting. la, lamina; medc, medulla cortex; lobc, lobula cortex. For details see text.

6. DISCUSSION

6.1. Gene regulatory network in neurogliogenesis acting downstream of *glial cells missing*

In *Drosophila*, the *glial cells missing* (*gcm*) gene acts as a genetic switch to control neuronal versus glial cell fate and is believed to initiate gliogenesis through the transcriptional activation of glial-specific target genes. However, the whole regulatory network is still poorly understood (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996; Jones, 2005). To identify the downstream genes regulated by *gcm*, in this thesis, two microarray experiments, which are based on either whole mount embryos or sorted neuroectodermal cells, were carried out (Egger et al., 2002; Montalta-He et al. in this thesis). In both cases, genome-wide oligonucleotide arrays were used to analyze differential expression in wild type embryos versus embryos in which *gcm* was misexpressed throughout the neuroectoderm. In the whole mount embryo-based microarray experiment, genome-wide gene expression levels were studied at two different developmental windows during embryogenesis. First, during a period of initial *gcm* action on the determination of neuro-glial cell progenitors (stage 11); and second, during a later period when glial cells have already differentiated (stage 15/16). At both stages, following *gcm* misexpression, significant changes in expression levels were found for hundreds of genes, which are referred to as candidate *gcm* downstream genes. Our prediction is that candidate genes identified at stage 11 are required for the determination of glial versus neuronal cell lineage, whereas candidates identified at the later stage are required for the maintenance of differentiated glial versus neuronal cells. In the following, some of these candidate genes are highlighted and classified in four groups.

First of all, genes that have previously been identified as glial specific show upregulated expression in the *gcm* gain-of-function situation. Prominent among these in early stages is *reversed polarity* (*repo*), which encodes a homeodomain transcription factor and is assumed to be a direct target of GCM (Akiyama et al., 1996). Another upregulated gene is *heartless* (*htl*) which encodes a FGF receptor and is expressed in glial progenitors and later in longitudinal glial cells. It is known that, in *htl* mutants, longitudinal glial cells fail to migrate to their final position in the VNC and are unable to enwrap axon bundles (Shishido et al., 1997; Condron, 1999). Moreover, the prominent *gcm*-induced upregulation of the *wrapper*

gene, which encodes an Ig superfamily member, is interesting. This was not expected since *wrapper* is prominently expressed in mesectoderm derived midline glial cells that are not dependent on *gcm* function. However, in this study we demonstrate that, late in embryogenesis, *wrapper* is also expressed in lateral glial cells and in glial cells supporting chordotonal organs in the PNS. These expression patterns are dependant on *gcm*. Therefore, *wrapper* is one of the few identified genes that are expressed in neuroectoderm as well as in mesectoderm derived glia. Mutational inactivation of *wrapper* leads to abnormalities in the commissural architecture and in the glial wrapping of commissural axons. The lack of close neuro-glia contact probably correlates with the increased cell death that was also observed in *wrapper* embryos (Noordermeer et al., 1998). The function of *wrapper* in *gcm* dependent lateral glia is currently unknown. In later embryonic stages *gcm* dependent upregulation was also found for *bangles and beads* (*bnb*). *bnb* mRNA can be detected in lateral glial cells and its expression is clearly dependent on the presence of *gcm*. BNB shares sequence similarities with the rat GAP-43 (growth associated protein or also known as neuromodulin) (Ng et al., 1989). The similarities, however, are confined to a region outside of the conserved amino-terminal domain characteristic for the vertebrate GAP-43 proteins. In addition, a second gene referred to as *igloo* (*igl*) has been identified in *Drosophila* that is more likely a homolog of vertebrate GAP-43 (Neel and Young, 1994). In mouse GAP-43 plays a crucial role in guiding the growth of axons and modulating the formation of new connections. It is almost exclusively observed in neuronal cells. However, expression was also detected in cultured astrocytes at an early stage of differentiation and in Schwann cells that are not in contact with axons (Benowitz and Routtenberg, 1997). The function of *bnb* in *Drosophila* is unknown and mutants are currently not available. Nevertheless, the prominent expression in lateral glial cells suggests a role in the glial differentiation pathway.

A second group of candidate genes that have previously been shown to play a role in neuronal development were detected as downregulated following *gcm* misexpression. Prominent among these is *scratch* (*scrt*), which is involved in early neuronal development. *scrt* is expressed in most or all neuronal precursor cells and encodes a predicted zinc finger transcription factor. Embryos lacking the function of *scrt* or *deadpan* (*dpn*, encoding a bHLH pan-neuronal transcription factor), reveal no gross abnormalities in neuronal development. However, concomitant inactivation of *scrt* and *dpn* leads to a severe reduction in the number of neurons. Furthermore, ectopic *scrt* expression results in precocious appearance of primary neuronal progenitor cells and the generation of extra neurons (Roark et al., 1995). During later

neuronal differentiation we detected clear decrease in transcript abundance for the gene *embryonic lethal, abnormal vision* (*elav*), which encodes an RNA-binding protein. *elav* is exclusively expressed in post-mitotic neurons and it is implicated in the post-transcriptional alternative splicing process for three neuronal specific protein isoforms (Koushika et al., 2000; Lisbin et al., 2001). It has been suggested that *elav* provides a vital function for neuronal differentiation and maintenance (Yao et al., 1993).

Another interesting group of candidate genes are those involved in the cell cycle regulation. Many recent observations have highlighted the functional cross talk between cell cycle regulation and cell fate determination (Cremisi et al., 2003). It has been shown in *Drosophila* that Ttk p69, besides repressing neuronal fate, also inhibits cell cycle progression by downregulation of the S-phase *cyclin E* (Badenhorst, 2001). Indeed, in our study we observe significant downregulation of *cyclin E* in late embryogenesis following *gcm* misexpression. However, for many other genes encoding cell cycle regulators we detect increased mRNA levels. Among these are several cyclin encoding genes as well as genes encoding kinases and structural elements involved in cell cycle progression.

Last but not least, for the majority of differentially regulated genes, the function is currently unknown and they have not been studied in any in vivo context. Therefore, a full appreciation and verification of all of these candidate *gcm* downstream genes and a comprehensive understanding of their roles in determination of glial versus neuronal fate will require a careful gene-by-gene analysis. Studies on the function of these genes will definitely shed some light on the key regulatory function of GCM in glia development. Considering that the single transcription factor GCM is sufficient to induce most aspects of gliogenesis in the *Drosophila* embryo and that neuroglial cell fate choice and differentiation is based on a complex genetic network, it is likely that GCM directly regulates the expression for an initial set of further transcription factors such as Repo, PntP1, Ttk p69 and signal transducers such as the FGF-receptor Htl. In turn, these genes regulate their own set of target genes, which are involved in different aspects of neurogliogenesis.

6.2. Experimental considerations of microarray analyses carried out in this thesis

Although we have succeeded in identifying a number of potential downstream genes of *gcm* in the whole mount embryo-based microarray experiment mentioned above, it is unlikely that

our study uncovers all of the genes that act downstream of *gcm* to induce glial cell fate. On the one hand, some potential genes might have been missed in our list. The reason could be, for example, that our transcript images are restricted to specific time points in nervous system development, and *gcm* may influence other targets at other stages. On the other hand, some candidate genes in our list might be false positive results due to the experimental design. For instance, the genetic overexpression of *gcm* may create an artificial situation in vivo, in which not all of the candidate downstream genes show changes in magnitude and direction of expression that correspond to their responses to *gcm* action under normal conditions. Therefore, although microarrays are powerful and efficient tools to quantify and compare gene expression on a large scale, microarray data alone, without in vivo verification, should be interpreted with great caution (Freeman et al., 2003). Moreover, careful attention to experimental design and data analysis is particularly important to avoid potential biases and improve the efficiency and reliability of microarray experiments. In this thesis, several aspects of experimental design and data analysis have been considered intensively.

The initial design of a microarray experiment needs high priority and can help to avoid validation problems and error finding. In general, microarray studies of the nervous system are inherently complicated by the diversity of cell populations and the lack of homogeneity in nervous tissue (Barlow and Lockhart, 2002; Griffin et al., 2003). Thus, cells of interest may comprise only a fraction of the entire tissue studied and genes expressed specifically in these cells might not be detectable when mRNA from whole animals is isolated. Also, a tissue-specific change in the expression of a given gene might be masked by uniform expression in other tissues. In consequence, averaging expression levels of entire tissue regions, or of entire embryos, may minimize or conceal even large expression changes that occur in small subpopulations of cells. This problem is aggravated in studies of neuronal development due to the small size of the embryonic nervous system and the difficulty in identifying the subpopulations of interest for dissection in embryos. In order to overcome this experimental obstacle, methods are needed that allow the isolation of specific neural cell subpopulations from embryos. In this thesis, we have developed and applied a microarray analysis based on genetic labeling techniques and magnetic cell sorting for isolating neuroectodermal cells from *Drosophila* embryos (Montalta-He et al. in this thesis). The high spatiotemporal specificity of the *GAL4-UAS* system was used to direct expression of mCD8-GFP, a molecular label suitable for magnetic cell isolation, exclusively to the neuroectoderm. Labeled cells were then dissociated and separated using magnetic cell sorting techniques, which permitted a high rate

of purification of viable cells from the neuroectoderm. Following microarray analysis on potential *gcm* downstream genes, we detected a total of 76 candidates. The relatively small number of genes identified in this cell sorting-based microarray experiment contrasts with the larger number (n = 242) of differentially regulated genes obtained in the comparable whole mount-based microarray experiment. It is likely that this is due to the use of more homogeneous target tissue in the cell sorting experiments. Further validation studies of genes identified as differentially expressed in the sorted cell-based microarray experiments revealed a high rate of in vivo verification of 82% (n = 14/17). Of these, 8 genes have for the first time been validated as *gcm* downstream genes by in situ hybridization, and two of these genes, *nerfin-1* and *aristaless*, have been shown to be involved in CNS or embryonic development (Schneitz et al., 1993; Stivers et al., 2000; Campbell, 2002; Tomancak et al., 2002). The function of the remaining 6 genes has not been determined yet. Thus, it will be important to know more about roles of these genes in neurogliogenesis and how they are regulated by the *gcm* gene. Given that the magnetic cell separation technique (MACS) only requires simple and economic experimental settings in comparison to other cell sorting techniques such as Laser Captured Microdissection (LCM), Fluorescent Associated Cell Separation (FACS), single cell transcript profiling and mRNA-tagging, this study should facilitate the application of microarray techniques in *Drosophila*.

The next aspect we considered in our microarray analysis is the reproducibility and the reliability of oligonucleotide array results. Several studies have stressed the importance of experiment repetitions and multiple independent trials that are necessary to obtain accurate data (Griffin et al., 2003). In the course of our studies we carried out at least four independent replicate experiments in order to perform statistical tests indicating the significance of differential expression. Moreover, it has been shown that different array platforms do not always result in the same outcome (Michaut et al., 2003). In this thesis, two different full genome Affymetrix GeneChips were used for global gene expression profiling in *Drosophila* embryos. One is a custom-designed *Drosophila* GeneChip (roDROMEGAa; Affymetrix Inc.) and the other is the commercial DrosGenome1 (Affymetrix, cat# 900 335). Both of them are based on the Release 1.0 of the *Drosophila* genome, but their way to select probes are different. Notably, the results achieved from these two different oligonucleotide arrays are not highly comparable. This is partially due to the incompatibility of the different platforms, but it is probably also a consequence of differences in target sequences representing individual gene products. Hence, careful tracing back from the microarray data to the original transcripts

represented on the individual GeneChip is important for getting reliable results from data analysis. Additionally, the sequence quality of available eukaryotic genomes does constantly improve and genome-wide annotation programs are in a process of re-evaluation. For example, in *Drosophila*, the number of protein-coding genes changed only minimally, from 13,601 genes in Release 1 to 13,474 genes in Release 2, and to 13,676 in Release 3. However, the re-annotation process accompanying Release 3 changed the majority of gene models, namely the information about UTRs, and exon-intron boundaries. Therefore, although the global picture of the number and distribution of transcription units remains similar compared to previous releases, the current annotation includes many changes in gene products (Misra et al., 2002). A recent effort to generate a *Drosophila* proteome database, FLYCAT (Brunner et al., 2005), will provide a more direct way to improve the current annotation of the *Drosophila* genome.

Once hundreds or thousands of genes are examined in microarray experiments, the appropriate mathematical tools need to be used to identify genes likely to be regulated in the manner of interest. One aspect of this process is to set an individual criterion for microarray data selection. In our genome-wide *gcm* downstream gene analyses, some genes encoding markers for lateral or peripheral glia were not judged to be upregulated by our data analysis simply because they were not picked up by the threshold filter we set. For example, in our analysis of stage 15/16 *sca-gcm* versus wild type embryos, three genes, *repo*, *loco* and *gliotactin*, were not considered to be upregulated in our microarray experiments because the normalized expression levels (represent by Avg Diff), the fold change levels (FCs) or the statistical significance levels (indicated by p-value) were below our threshold filter values. Although a standard data selection criterion does not exist for all microarray analyses, getting the most optimal one for a particular experiment can improve the efficiency and reliability of microarray analysis. Hence, it is reasonable to evaluate the optional criteria by using in vitro or in vivo experimental verifications in advance. In our case, in situ hybridization and antibody immunostaining was used to verify the potential *gcm* downstream genes. Based on the results of more than 60 candidate genes, the high value (above 50%) of in situ confirmation can be achieved by setting a threshold of Avg-Diff for both wild type and *gcm* misexpression conditions (i.e. above or equal to 50 in both conditions) as well as using relatively high FCs (i.e. above 2-fold) and low p-value (i.e. $p \leq 0.01$) in the selection filter. Following the selection of microarray data, the next step is validation, which requires measuring the expression of specific genes of interest using independent methods and

independent RNA samples (Griffin et al., 2003). This is crucial for further improving and substantiating the results of microarray experiments. However, a general notion is that, at present, alternative techniques are not yet available to validate large-scale differential gene expression results at genomic levels. Several possibilities are available for confirming changes in expression levels and patterns for single or a small number of genes. We found that RT-PCR allows comparing quantitatively expression levels for many genes in accurate resolution and in a reasonable timeframe. In most of our studies, the most useful technique in visualizing and validating changes in mRNA expression was whole-mount in situ hybridization. However, due to the present lack of appropriate high throughput protocols, this is a tedious and time-consuming method. Furthermore, although in situ hybridization is a valuable method to visualize changes in spatial and temporal expression patterns that might lead directly to the function of the gene (Barlow and Lockhart, 2002), it is not suitable to validate small changes in expression levels i.e. in the same group of cells. Taken together, biological characterization of genes identified by microarrays requires great attention to experimental design, quality control and methods of analysis.

6.3. The *egghead* gene encodes a glycosyltransferase involved in compartmentalization of the optic lobe

The *egghead* (*egh*) gene is one of the potential *gcm* downstream targets identified in our microarray analysis. It was upregulated at both early and late stages with relative expression level changes of 1.9-fold when *gcm* was misexpressed throughout the neuroectoderm. This was also verified by using in situ hybridization. In wild type embryos, the expression of *egh* in the CNS is low, whereas in *gcm* gain-of-function embryos, the expression of *egh* in the CNS is clearly upregulated. The *egh* gene comprises 4 exons and 3 introns and extends over approximately 10kb on the X chromosome. So far, it is known that there are three or four different transcripts for *egh*. Northern blot analysis with tissue specific transcripts shows that the longest *egh* transcript which contains all 4 exons is enriched in the adult brain (Martin Hollmann, personal communication). These expression data suggest that *egh* might be involved in CNS development and be regulated by *gcm*. Furthermore, in silico studies have identified 10 GCM binding sites within the 3kb intron 2 of the *egh* gene. This suggests that *egh* might be a direct target of *gcm*. However, this hypothesis still needs to be carefully studied in vivo.

The function of *egh* was initially studied in oogenesis. There, *egh* is essential for the formation and maintenance of the follicular epithelium as well as for the migration and maintenance of the border cells. Thus, *egh* has been proposed to regulate the oocyte-follicle cell adhesion system (Goode et al., 1996). Moreover, *egh* has been characterized as a neurogenic gene because embryos derived from *egh* germ line clone females show a Notch-like neurogenic phenotype, that is, the nervous system is expanded in regions where the epidermal layer is missing. In contrast, egh^{-}/Y embryos derived from *egh* heterozygous females display a largely normal embryonic CNS. Such animals may occasionally reach the adult stage and show a severe neurodegeneration phenotype in the medulla and lobula complex (Matthias Soller, personal communication). Taken together, it seems that *egh* plays various roles at different stages of *Drosophila* development. In this thesis, we analyzed the role of *egh* in the development of *Drosophila* visual system (Fan et al. in this thesis). The genetic analysis involving mosaics demonstrates that *egh* is required for a compartment boundary between lamina glia and lobula cortex. Moreover, this requirement of *egh* is not in the eye or in the neurons and glia of the lamina, but instead, is restricted to cells of the lobula complex primordium during larval development. In the absence of *egh*, perturbation of glial sheaths occurs at the boundary region delimiting lamina glia and lobula cortex, and inappropriate invasion of lobula cortex cells across the boundary region disrupts the pattern of lamina glia resulting in inappropriate R1-R6 innervation. These results clearly indicate the crucial role of the lamina/lobula cortex boundary in photoreceptor axon targeting and highlight the importance of *egh* in the compartmentalization of *Drosophila* visual centers. Our genetic analysis also provides an entry point for further intriguing questions. How might the *egh* gene contribute to compartmentalization of the optic lobe? What are the molecular mechanisms for the compartment boundary establishment and maintenance?

Analysis of the protein domain and structural motifs suggests that *egh* encodes a Golgi/ER-located glycosyltransferase (Wandall et al., 2003). In vitro glycosyltransferase assays and recent in vivo analysis showed that *egh* was capable of forming the precursor glycospingolipid substrate, mactosylceramide, and was essential for glycospingolipid biosynthesis (Wandall, et al., 2005). In vertebrates, glycospingolipids have functions in relation to cell adhesion, growth, regulation, differentiation, cell interaction, recognition and signaling (Watts, 2003). Interestingly, glycolipids were shown recently to serve as host cell receptors for crystal toxin in *C. elegans* (Griffitts et al., 2005). Many studies suggest that particularly ordered lipid environments are enriched with certain membrane proteins such as

caveolins, src-family kinases, and glycosylphosphatidylinositol (GPI) – anchored proteins. Thus, the lipid composition of the plasma membrane is believed to provide suitable microenvironments to enable selective protein-protein interactions as well as local initiation of signal transduction (Simons and Toomre, 2000; Tsui-Pierchala et al., 2002). Despite differences in the chemical structure of their lipids, *Drosophila* membranes contain microdomains with a similar protein and lipid composition as their mammalian counterparts. Signaling and polarized intracellular transport of Hedgehog had been shown to associate with these domains in *Drosophila* embryos (Rietveld S. et al. 1999). This suggests that lipid composition and function is preserved from flies to vertebrates. Although the existence of cholesterol- and sphingolipid-enriched membrane microdomains (lipid rafts) remains a contentious issue (Munro, 2003), lipid microdomains were recently shown to play a direct role in organizing spatial signaling during cell chemotaxis and axon guidance by concentrating the gradient-sensing machinery at the leading cell edge (Gomez-Mouton et al., 2004; Guirland et al., 2004). Thus, it is possible that in *Drosophila*, *egh* regulates the organization of lipid composition in the plasma membrane by controlling the biosynthesis of glycospingolipids. At least cells in the anterior part of the lobula complex, which is adjacent to the developing lamina, may need suitable lipid microenviroments to mediate specific signal cues for the maintenance of the boundary between lamina glia and lobula cortex or for the control of the proper movement of the lobula distal cells during optic lobe development. Hence, further insight into the molecular pathways that mediate *egh* action in compartmentalization may be obtained by the isolation of glycosphingolipid-associated cell surface molecules.

However, considering that the Egh protein is Golgi/ER-localized, an alternative interpretation of our data is that *egh* affects compartmentalization signal cues through the modification of protein glycosylation. In *Drosophila*, glycosyltransferase gene *fringe* and O-fucosyltransferase gene *Ofut1* have been shown to regulate ligand-receptor interactions by modifying the EGF repeats in Notch signaling (Bruckner et al., 2000; Okajima and Irvine, 2002). Interestingly, during oogenesis, *egh* is proposed to be involved in *Notch*-mediated epithelial development. It also interacts with the Egfr signaling pathway (Goode et al., 1992; Goode et al., 1996). Further detailed analysis or isolation of the molecules involved in the boundary formation or maintenance will help understand how a glycosylation pathway is involved in the compartmentalization of the *Drosophila* brain.

7. REFERENCES

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Akam, M. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1-22.

Akiyama, Y., Hosoya, T., Poole, A. M. and Hotta, Y. (1996). The *gcm*-motif: a novel DNA-binding motif conserved in *Drosophila* and mammals. *Proc. Natl. Acad. Sci. U S A* **93**, 14912-14916.

Akiyama-Oda, Y., Hosoya, T. and Hotta, Y. (1998). Alteration of cell fate by ectopic expression of *Drosophila glial cells missing* in non-neural cells. *Dev. Genes Evol.* **208**, 578-585.

Akiyama-Oda, Y., Hosoya, T. and Hotta, Y. (1999). Asymmetric cell division of thoracic neuroblast 6-4 to bifurcate glial and neuronal lineage in *Drosophila*. *Development* **126**, 1967-1974.

Akiyama-Oda, Y., Hotta, Y., Tsukita, S. and Oda, H. (2000). Distinct mechanisms triggering glial differentiation in *Drosophila* thoracic and abdominal neuroblasts 6-4. *Dev. Biol.* **222**, 429-439.

Alfonso, T. B. and Jones, B. W. (2002). *gcm2* promotes glial cell differentiation and is required with *glial cells missing* for macrophage development in *Drosophila*. *Dev. Biol.* **248**, 369-383.

Altshuller, Y., Copeland, N. G., Gilbert, D. J., Jenkins, N. A. and Frohman, M. A. (1996). Gcm1, a mammalian homolog of *Drosophila glial cells missing*. *FEBS Lett.* **393**, 201-204.

Anderson, D. J. (2001). Stem cells and pattern formation in the nervous system: the possible versus the actual. *Neuron* **30**,19-35.

Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J. and Johnson, W. A. (1995). *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* **9**, 123-137.

Aoyagi, N. and Wassarman, D. A. (2000). Genes encoding *Drosophila melanogaster* RNA polymerase II general transcription factors: diversity in TFIIA and TFIID components contributes to gene-specific transcriptional regulation. *J. Cell. Biol.* **150**, F45-50.

Araujo, S. J. and Tear, G. (2003). Axon guidance mechanisms and molecules: lessons from invertebrates. *Nat. Rev. Neurosci.* **4**, 910-922.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Ashburner, M. (1989). *Drosophila: A Laboratory Manual.* pp. 399-402. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T. et al. (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25-29.

Badenhorst, P. (2001). Tramtrack controls glial number and identity in the *Drosophila* embryonic CNS. *Development* **128**, 4093-4101.

Bardin, A. J., Le Borgne, R. and Schweisguth, F. (2004). Asymmetric localization and function of cell-fate determinants: a fly's view. *Curr. Opin. Neurobiol.* **14**, 6-14.

Barlow, C. and Lockhart, D. J. (2002). DNA arrays and neurobiology--what's new and what's next? *Curr. Opin. Neurobiol.* **12**, 554-561.

Basler, K. and Hafen, E. (1988). Control of photoreceptor cell fate by the sevenless protein requires a functional tyrosine kinase domain. *Cell* **54**, 299-311.

Baumgartner, S., Martin, D., Hagios, C. and Chiquet-Ehrismann, R. (1994). *ten^{m}*, a *Drosophila* gene related to tenascin, is a new pair-rule gene. *EMBO J.* **13**, 3728-3740.

Bausenwein, B., Dittrich, A. P. M. and Fischbach, K. F. (1992). The optic lobe of *Drosophila melanogaster*. II. Sorting of retinotopic pathways in the medulla. *Cell Tussue Res.* **267**, 17-28.

Benowitz, L. I. and Routtenberg, A. (1997). GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends Neurosci.* **20**, 84-91.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in *Drosophila*. *Dev. Cell* **3**, 221-231.

Bernardoni, R., Kammerer, M., Vonesch, J. L. and Giangrande, A. (1999). Gliogenesis depends on *glide*/*gcm* through asymmetric division of neuroglioblasts. *Dev. Biol.* **216**, 265-275.

Bernardoni, R., Miller, A. A. and Giangrande, A. (1998). Glial differentiation does not require a neural ground state. *Development* **125**, 3189-3200.

Bernardoni, R., Vivancos, V. and Giangrande, A. (1997). *glide/gcm* is expressed and required in the scavenger cell lineage. *Dev. Biol.* **191**, 118-130.

Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

Betschinger, J. and Knoblich, J. A. (2004). Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. *Curr. Biol.* **14**, R674-685.

Bhat, K. M. (1996). The patched signaling pathway mediates repression of *gooseberry* allowing neuroblast specification by *wingless* during *Drosophila* neurogenesis. *Development* **122**, 2921-2932.

Bhat, K. M. (1999). Segment polarity genes in neuroblast formation and identity specification during *Drosophila* neurogenesis. *Bioessays* **21**, 472-485.

Bhat, K. M. and Schedl, P. (1997). Requirement for *engrailed* and *invected* genes reveals novel regulatory interactions between *engrailed/invected*, *patched*, *gooseberry* and *wingless* during *Drosophila* neurogenesis. *Development* **124**, 1675-1688.

Bhat, K. M., van Beers, E. H. and Bhat, P. (2000). Sloppy paired acts as the downstream target of Wingless in the *Drosophila* CNS and interaction between *sloppy paired* and *gooseberry* inhibits *sloppy paired* during neurogenesis. *Development* **127**, 655-665.

Bonkowsky, J. L., Yoshikawa, S., O'Keefe, D. D., Scully, A. L. and Thomas, J. B. (1999). Axon routing across the midline controlled by the *Drosophila* Derailed receptor. *Nature* **402**, 540-544.

Booth, G. E., Kinrade, E. F. and Hidalgo, A. (2000). Glia maintain follower neuron survival during *Drosophila* CNS development. *Development* **127**, 237-244.

Bossing, T., Udolph, G., Doe, C. Q. and Technau, G. M. (1996). The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. *Dev. Biol.* **179**, 41-64.

Bourouis, M., Heitzler, P., el Messal, M. and Simpson, P. (1989). Mutant *Drosophila* embryos in which all cells adopt a neural fate. *Nature* **341**, 442-444.

Boyan, G., Therianos, S., Williams, J. L. and Reichert, H. (1995). Axogenesis in the embryonic brain of the grasshopper *Schistocerca gregaria*: an identified cell analysis of early brain development. *Development* **121**, 75-86.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Broadus, J., Skeath, J. B., Spana, E. P., Bossing, T., Technau, G., and Doe, C. Q. (1995). New neuroblast markers and the origin of the aCC/pCC neurons in the *Drosophila* central nervous system. *Mec. Dev.* **53**, 393-402.

Brody, T. and Odenwald, W. F. (2000). Programmed transformations in neuroblast gene expression during *Drosophila* CNS lineage development. *Dev. Biol.* **226**, 34-44.

Brody, T. and Odenwald, W. F. (2002). Cellular diversity in the developing nervous system: a temporal view from *Drosophila*. *Development* **129**, 3763-3670.

Brown, P. O. and Botstein, D. (1999). Exploring the new world of the genome with DNA microarrays. *Nat. Genet.* **21**, 33-37.

Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-415.

Brunner, E., Kregenow, F., Deutsch, E., Eng, J., Pedrioli, P., Donohoe, S., Yi, E., Purvine, S., Vaughan, K., Goodlett, D., Aebersold, R., and Hafen, E. (2005). FLYCAT - Towards a *Drosophila* proteome database. Symposia S0104 in a joint annual meeting of the Union of the Swiss Societies for Experimental Research, Swiss Society for Neuroscience and Swiss Society of Biological Psychiatry.

Bryant, Z., Subrahmanyan, L., Tworoger, M., LaTray, L., Liu, C. R., Li, M. J., van den Engh, G. and Ruohola-Baker, H. (1999). Characterization of differentially expressed genes in purified *Drosophila* follicle cells: toward a general strategy for cell type-specific developmental analysis. *Proc. Natl. Acad. Sci. USA* **96**, 5559-5564.

Bryant, Z., Subrahmanyan, L., Tworoger, M., LaTray, L., Liu, C. R., Li, M. J., van den Engh, G. and Ruohola-Baker, H. (1999). Characterization of differentially expressed genes in purified *Drosophila* follicle cells: toward a general strategy for cell type-specific developmental analysis. *Proc. Natl. Acad. Sci. USA* **96**, 5559-5564.

Cafferty, P., Yu, L. and Rao, Y. (2004). The receptor tyrosine kinase Off-track is required for layer-specific neuronal connectivity in *Drosophila*. *Development* **131**, 5287-5295.

Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* **120**, 2957-2966.

Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* **120**, 2957-2966.

Campos-Ortega, J. and Hartenstein, V. (1997). The Embryonic Development of *Drosophila melanogaster*. Heidelberg: Springer.

Campuzano, S. and Modolell, J. (1992). Patterning of the *Drosophila* nervous system: the *achaete-scute* gene complex. *Trends Genet.* **8**, 202-208.

Casso, D., Ramirez-Weber, F. and Kornberg, T. B. (2000). GFP-tagged balancer chromosomes for *Drosophila melanogaster*. *Mech. Dev.* **91**, 451-454.

Celniker, S. E., Wheeler, D. A., Kronmiller, B., Carlson, J. W., Halpern, A., Patel, S., Adams, M., Champe, M., Dugan, S. P., Frise, E. et al. (2002). Finishing a whole-genome shotgun: release 3 of the *Drosophila melanogaster* euchromatic genome sequence. *Genome Biol.* **3**, RESEARCH0079.

Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev. Cell* **6**, 539-550.

Chia, W. and Yang, X. (2002). Asymmetric division of *Drosophila* neural progenitors. *Curr. Opin. Genet. Dev.* **12**, 459-464.

Choi, K. W. and Benzer, S. (1994). Migration of glia along photoreceptor axons in the developing *Drosophila* eye. *Neuron* **12**, 423-431.

Chotard, C. and Salecker, I. (2004). Neurons and glia: team players in axon guidance. *Trends Neurosci.* **27**, 655-661.

Chu-LaGraff, Q. and Doe, C. Q. (1993). Neuroblast specification and formation regulated by *wingless* in the *Drosophila* CNS. *Science* **261**, 1594-1597.

Clandinin, T. R. and Zipursky, S. L. (2000). Afferent growth cone interactions control synaptic specificity in the *Drosophila* visual system. *Neuron* **28**, 427-436.

Clandinin, T. R. and Zipursky, S. L. (2002). Making connections in the fly visual system. *Neuron* **35**, 827-841.

Clandinin, T. R., Lee, C. H., Herman, T., Lee, R. C., Yang, A. Y., Ovasapyan, S. and Zipursky, S. L. (2001). *Drosophila* LAR regulates R1-R6 and R7 target specificity in the visual system. *Neuron* **32**, 237-248.

Condron, B. (1999). Spatially discrete FGF-mediated signalling directs glial morphogenesis. *Development* **126**, 4635-4641.

Condron, B. G., Patel, N. H. and Zinn, K. (1994). Engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system. *Neuron* **13**, 541-554.

Connell-Crowley, L., Le Gall, M., Vo, D. J. and Giniger, E. (2000). The cyclin-dependent kinase Cdk5 controls multiple aspects of axon patterning in vivo. *Curr. Biol.* **10**, 599-602.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene *kr* encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-1034.

Cornell, R. A. and Ohlen, T. V. (2000). Vnd/nkx, ind/gsh, and msh/msx: conserved regulators of dorsoventral neural patterning? *Curr. Opin. Neurobiol.* **10**, 63-71.

Cremisi, F., Philpott, A. and Ohnuma, S. (2003). Cell cycle and cell fate interactions in neural development. *Curr. Opin. Neurobiol.* **13**, 26-33.

Crews, S. T., Thomas, J. B. and Goodman, C. S. (1988). The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* **52**, 143-151.

Cubadda, Y., Heitzler, P., Ray, R. P., Bourouis, M., Ramain, P., Gelbart, W., Simpson, P. and Haenlin, M. (1997). *u-shaped* encodes a zinc finger protein that regulates the proneural genes *achaete* and *scute* during the formation of bristles in *Drosophila*. *Genes Dev.* **11**, 3083-3095.

Daffre, S., Kylsten, P., Samakovlis, C. and Hultmark, D. (1994). The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. *Mol.Gen. Genet.* **242**, 152-162.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet.* **15**, 320-326.

Datta, S. (1995). Control of proliferation activation in quiescent neuroblasts of the *Drosophila* central nervous system. *Development* **121**, 1173-1182.

Dearborn, R., Jr. and Kunes, S. (2004). An axon scaffold induced by retinal axons directs glia to destinations in the *Drosophila* optic lobe. *Development* **131**, 2291-2303.

Delidakis, C. and Artavanis-Tsakonas, S. (1992). The Enhancer of split [E(spl)] locus of *Drosophila* encodes seven independent helix-loop-helix proteins. *Proc. Natl. Acad. Sci. U S A* **89**, 8731-8735.

DeSimone, S. M. and White, K. (). The *Drosophila erect wing* gene, which is important for both neuronal and muscle development, encodes a protein which is similar to the sea urchin P3A2 DNA binding protein. *Mol. Cell Biol.* **13**, 3641-3649.

Deyholos, M. K. and Galbraith, D. W. (2001). High-density microarrays for gene expression analysis. *Cytometry* **43**, 229-238.

Doe, C. Q. (1992). Molecular markers for identified neuroblasts and ganglion mother cells in the *Drosophila* central nervous system. *Development* **116**, 855-863.

Doe, C. Q. and Skeath, J. B. (1996). Neurogenesis in the insect central nervous system. *Curr. Opin. Neurobiol.* **6**, 18-24.

Doe, C. Q., Smouse, D. and Goodman, C. S. (1988). Control of neuronal fate by the *Drosophila* segmentation gene *even-skipped*. *Nature* **333**, 376-378.

Edenfeld, G., Stork, T. and Klambt, C. (2005). Neuron-glia interaction in the insect nervous system. *Curr. Opin. Neurobiol.* **15**, 34-39.

Egger, B., Leemans, R., Loop, T., Kammermeier, L., Fan, Y., Radimerski, T., Strahm, M. C., Certa, U. and Reichert, H. (2002). Gliogenesis in *Drosophila*: genome-wide analysis of downstream genes of *glial cells missing* in the embryonic nervous system. *Development* **129**, 3295-3309.

Enomoto H. (2005). Regulation of neural development by glial cell line-derived neurotrophic factor family ligands. *Anat. Sci. Int.* **80**, 42-52.

Eule, E., Tix, S. and Fischbach, K. F. (1995). Glial cells in the optic lobe of the *Drosophila melanogaster*. Flybrain on-line [http://www.flybrain.org]. Accession number: PP00004.

Ferguson, E. L. and Anderson, K. V. (1992). Decapentaplegic acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451-461.

Fischbach, K. F. and Dittrich A. P. M. (1989). The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. *Cell Tissue Res.* **258**, 441-475.

Fleming, R. J. (1998). Structural conservation of Notch receptors and ligands. *Semin. Cell Dev. Biol.* **9**, 599-607.

Follette, P. J. and O'Farrell, P. H. (1997). Cdks and the *Drosophila* cell cycle. *Curr. Opin. Genet. Dev.* **7**, 17-22.

Francis, N. J. and Kingston, R. E. (2001). Mechanisms of transcriptional memory. *Nat. Rev. Mol. Cell. Biol.* **2**, 409-421.

Frank, D. J., Tyree, C. M., George, C. P. and Kadonaga, J. T. (1995). Structure and function of the small subunit of TFIIF (RAP30) from *Drosophila* melanogaster. *J. Biol. Chem.* **270**, 6292-6297.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87**, 651-660.

Freeman, M. R., Delrow, J., Kim, J., Johnson, E. and Doe, C. Q. (2003). Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. *Neuron* **38**, 567-580.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* **26**, 383-394.

Gage, F. H. (2000). Mammalian neural stem cells. *Science* **287**, 1433-1438.

Garrity, P. A., Lee, C. H., Salecker, I., Robertson, H. C., Desai, C. J., Zinn, K. and Zipursky, S. L. (1999). Retinal axon target selection in *Drosophila* is regulated by a receptor protein tyrosine phosphatase. *Neuron* **22**, 707-717.

Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996). *Drosophila* photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* **85**, 639-650.

Ghosh, A. and Shatz, C. J. (1993). A role for subplate neurons in the patterning of connections from thalamus to neocotex. *Development* **117**, 1031-1047.

Ghysen, A. and Dambly-Chaudiere, C. (1989). Genesis of the *Drosophila* peripheral nervous system. *Trends Genet.* **5**, 251-255.

Giesen, K., Hummel, T., Stollewerk, A., Harrison, S., Travers, A. and Klambt, C. (1997). Glial development in the *Drosophila* CNS requires concomitant activation of glial and repression of neuronal differentiation genes. *Development* **124**, 2307-2316.

Gomez-Mouton, C., Abad, J. L., Mira, E., Jimenez-Baranda, S., Barber, D. F., Carrera, A. C. and Manes, S. (2004). Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. *J. Cell Biol.* **164**, 759-768.

Goode, S., Melnick, M., Chou, T. B. and Perrimon, N. (1996). The neurogenic genes *egghead* and *brainiac* define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis. *Development* **122,** 3863-3879.

Goode, S., Wright, D. and Mahowald, A. P. (1992). The neurogenic locus *brainiac* cooperates with the *Drosophila* EGF receptor to establish the ovarian follicle and to determine its dorsal-ventral polarity. *Development* **116**, 177-192.

Granderath, S. and Klambt, C (1999). Glia development in the embryonic CNS of *Drosophila*. *Curr. Opin. Neurobiol.* **9**, 531-536.

Granderath, S., Bunse, I. and Klambt, C. (2000). *gcm* and *pointed* synergistically control glial transcription of the *Drosophila* gene *loco*. *Mech. Dev.* **91**, 197-208.

Granderath, S., Stollewerk, A., Greig, S., Goodman, C. S., O'Kane, C. J. and Klambt, C. (1999). *loco* encodes an RGS protein required for *Drosophila* glial differentiation. *Development* **126**, 1781-1791.

Griffin, R. S., Mills, C. D., Costigan, M. and Woolf, C. J. (2003). Exploiting microarrays to reveal differential gene expression in the nervous system. *Genome Biol.* **4**, 105.

Griffitts, J. S., Haslam, S. M., Yang, T., Garczynski, S. F., Mulloy, B., Morris, H., Cremer, P. S., Dell, A., Adang, M. J. and Aroian, R. V. (2005). Glycolipids as receptors for Bacillus thuringiensis crystal toxin. *Science* **307**, 922-925.

Guirland, C., Suzuki, S., Kojima, M., Lu, B. and Zheng, J. Q. (2004). Lipid rafts mediate chemotropic guidance of nerve growth cones. *Neuron* **42**, 51-62.

Hacker, U., Grossniklaus, U., Gehring, W. J. and Jackle, H. (1992). Developmentally regulated *Drosophila* gene family encoding the fork head domain. *Proc. Natl. Acad. Sci. U S A* **89**, 8754-8758.

Hahn, M. and Jackle, H. (1996). *Drosophila goosecoid* participates in neural development but not in body axis formation. *EMBO J.* **15**, 3077-3084.

Hahnlein, I. and Bicker, G. (1996). Morphology of neuroglia in the antennal lobes and mushroom bodies of the brain of the honeybee. *J. Comp. Neurol.* **367**, 235-245.

Halter, D. A., Urban, J., Rickert, C., Ner, S. S., Ito, K., Travers, A. A. and Technau, G. M. (1995). The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* **121**, 317-332.

Heberlein, U. and Rubin, G. M. (1990). Structural and functional comparisons of the *Drosophila virilis* and *Drosophila melanogaster* rough genes. *Proc. Natl. Acad. Sci. USA* **87**, 5916-5920.

Henry, G. L., Zito, K. and Dubnau, J. (2003). Chipping away at brain function: mining for insights with microarrays. *Curr. Opin. Neurobiol.* **13**, 570-576.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of *Drosophila*. *Development* **125**, 1579-1589.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **198**, 264-274.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F. and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. *Development* **127**, 2515-2522.

Hosoya, T., Takizawa, K., Nitta, K. and Hotta, Y. (1995). *glial cells missing*: a binary switch between neuronal and glial determination in *Drosophila*. *Cell* **82**, 1025-1036.

Huang, Z. and Kunes, S. (1998). Signals transmitted along retinal axons in *Drosophila*: Hedgehog signal reception and the cell circuitry of lamina cartridge assembly. *Development* **125**, 3753-3764.

Hummel, T., Attix, S., Gunning, D. and Zipursky, S. L. (2002). Temporal control of glial cell migration in the *Drosophila* eye requires *gilgamesh*, *hedgehog*, and eye specification genes. *Neuron* **33**, 193-203.

Hummel, T., Schimmelpfeng, K., Klambt, C. (1999a). Commissure formation in the embryonic CNS of *Drosophila*: I Identification of the required gene functions. *Dev. Biol.* **209**, 381–398.

Hummel, T., Schimmelpfeng, K., Klambt, C. (1999b). Commissure formation in the embryonic CNS of *Drosophila*: II Function of the different midline cells. *Development* **126**, 771–779.

Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu. Rev. Cell Dev. Biol.* **17**, 189-214.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

Ito, K., Urban, J. and Technau, G. M. (1995). Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Roux's Arch. Dev. Biol.* **204**, 284-307.

Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M. and Uemura, T. (1997). Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the *Drosophila* embryonic CNS. *Neuron* **19**, 77-89.

Iwasaki, Y., Hosoya, T., Takebayashi, H., Ogawa, Y., Hotta, Y. and Ikenaka, K. (2003). The potential to induce glial differentiation is conserved between *Drosophila* and mammalian *glial cells missing* genes. *Development* **130**, 6027-6035.

Jennings, B., Preiss, A., Delidakis, C. and Bray, S. (1994). The Notch signalling pathway is required for Enhancer of split bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* **120**, 3537-3548.

Ji, W., Cai, L., Wright, M. B., Walker, G., Salgam, P., Vater, A. and Lindpaintner, K. (2000). Preservation of gene expression ratios among multiple complex cDNAs after PCR amplification: application to differential gene expression studies. *J. Struct. Funct. Genomics* **1**, 1-7.

Johnson, K. G., Ghose, A., Epstein, E., Lincecum, J., O´Connor, M. B. and Van Vactor, D. (2004). Axonal heparan sulfate proteoglycans regulate the distribution and efficiency of the repellent Slit during midline axon guidance. *Curr. Biol.* **14**, 499-504.

Jones, B. W. (2001). Glial cell development in the *Drosophila* embryo. *Bioessays* **23**, 877-887.

Jones, B. W. (2005). Transcriptional control of glial cell development in *Drosophila*. *Dev. Biol.* **278**, 265-273.

Jones, B. W., Abeysekera, M., Galinska, J. and Jolicoeur, E. M. (2004). Transcriptional control of glial and blood cell development in *Drosophila*: cis-regulatory elements of *glial cells missing*. *Dev. Biol.* **266**, 374-387.

Jones, B. W., Fetter, R. D., Tear, G. and Goodman, C. S. (1995). *glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell* **82**, 1013-1023.

Judd, B. H., Shen, M. W. and Kaufman, T. C. (1972). The anatomy and function of a segment of the X chromosome of *Drosophila melanogaster*. *Genetics* **71**, 139-156.

Justice, N., Roegiers, F., Jan, L. Y. and Jan, Y. N. (2003). Lethal giant larvae acts together with Numb in Notch inhibition and cell fate specification in the *Drosophila* adult sensory organ precursor lineage. *Curr. Biol.* **13**, 778-783.

Kain, K. C., Orlandi P. A. and Lanar D. E. (1991). Universal promoter for gene expression without cloning: expression-PCR. *Biotechniques* **10**, 366-374.

Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S. J. and Odenwald, W. F. (1998). Regulation of POU genes by *castor* and *hunchback* establishes layered compartments in the *Drosophila* CNS. *Genes Dev.* **12**, 246-260.

Kaminker, J. S., Canon, J., Salecker, I. and Banerjee, U. (2002). Control of photoreceptor axon target choice by transcriptional repression of Runt. *Nat. Neurosci.* **5**, 746-750.

Kammerer, M. and Giangrande, A. (2001). Glide2, a second glial promoting factor in *Drosophila melanogaster*. *Embo J.* **20**, 4664-4673.

Kammerer, M., Pirola, B., Giglio, S. and Giangrande, A. (1999). GCMB, a second human homolog of the fly *glide/gcm* gene. *Cytogenet. Cell Genet.* **84**, 43-47.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the *Drosophila* Pax6 genes *eyeless* and *twin of eyeless* in embryonic central nervous system development. *Mech. Dev.* **103**, 71-78.

Kanemura, Y., Hiraga, S., Arita, N., Ohnishi, T., Izumoto, S., Mori, K., Matsumura, H., Yamasaki, M., Fushiki, S. and Yoshimine, T. (1999). Isolation and expression analysis of a novel human homologue of the *Drosophila glial cells missing* (*gcm*) gene. *FEBS Lett.* **442**, 151-156.

Kania, A., Han, P. L., Kim, Y. T. and Bellen, H. (1993). *neuromusculin*, a *Drosophila* gene expressed in peripheral neuronal precursors and muscles, encodes a cell adhesion molecule. *Neuron* **11**, 673-687.

Keene, J. D. (2001). Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. *Proc. Natl. Acad. Sci. U S A* **98**, 7018-7024.

Kim, J., Jones, B. W., Zock, C., Chen, Z., Wang, H., Goodman, C. S. and Anderson, D. J. (1998). Isolation and characterization of mammalian homologs of the *Drosophila* gene *glial cells missing*. *Proc. Natl. Acad. Sci. USA* **95**, 12364-12369.

Klaes, A., Menne, T., Stollewerk, A., Scholz, H. and Klambt, C. (1994). The Ets transcription factors encoded by the *Drosophila* gene *pointed* direct glial cell differentiation in the embryonic CNS. *Cell* **78**, 149-160.

Klambt, C. (1993). The *Drosophila* gene *pointed* encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* **117**, 163-176.

Klambt, C. and Goodman, C. S. (1991). The diversity and pattern of glia during axon pathway formation in the *Drosophila* embryo. *Glia* **4**, 205-213.

Klambt, C., Hummel, T., Granderath, S. and Schimmelpfeng, K. (2001). Glial cell development in *Drosophila*. *Int. J. Dev. Neurosci.* **19**, 373-378.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R. and Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77**, 107-120.

Koushika, S. P., Soller, M. and White, K. (2000). The neuron-enriched splicing pattern of *Drosophila erect wing* is dependent on the presence of ELAV protein. *Mol. Cell Biol.* **20**, 1836-1845.

Le Borgne, R. and Schweisguth, F. (2003). Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. *Dev. Cell* **5**, 139-148.

Le Borgne, R., Bellaiche, Y. and Schweisguth, F. (2002). *Drosophila* E-cadherin regulates the orientation of asymmetric cell division in the sensory organ lineage. *Curr. Biol.* **12**, 95-104.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000). Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science* **288**, 146-149.

Lee, C. H., Herman, T., Clandinin, T. R., Lee, R. and Zipursky, S. L. (2001). N-cadherin regulates target specificity in the *Drosophila* visual system. *Neuron* **30**, 437-450.

Lee, K. S., Oh, Y., Baek, G., Yoon, J., Han, K., Cho, N. and Baek, K. (1997). Analysis of the structure and expression fo the *TFIIB* gene in *Drosophila melanogaster*. *Mol. Cells* **7**, 374-379.

Lee, R. C., Clandinin, T. R., Lee, C. H., Chen, P. L., Meinertzhagen, I. A. and Zipursky, S. L. (2003). The protocadherin Flamingo is required for axon target selection in the *Drosophila* visual system. *Nat. Neurosci.* **6**, 557-563.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.

Leemans, R., Loop, T., Egger, B., He, H., Kammermeier, L., Hartmann, B., Certa, U., Reichert, H. and Hirth, F. (2001). Identification of candidate downstream genes for the homeodomain transcription factor Labial in *Drosophila* through oligonucleotide-array transcript imaging. *Genome Biol.* **2**, RESEARCH0015.

Levine, A., Bashan-Ahrend, A., Budai-Hadrian, O., Gartenberg, D., Menasherow, S. and Wides, R. (1994). *Odd Oz*: a novel *Drosophila* pair rule gene. *Cell* **77**, 587-598.

Lichtneckert, R. and Reichert, H. (2005). Insights into the urbilaterian brain: conserved genetic patterning mechanisms in insect and vertebrate brain development. *Heredity* **94**, 465-477.

Lipshutz, R. J., Fodor, S. P., Gingeras, T. R. and Lockhart, D. J. (1999). High density synthetic oligonucleotide arrays. *Nat. Genet.* **21**, 20-24.

Lipshutz, R. J., Fodor, S. P., Gingeras, T. R. and Lockhart, D. J. (1999). High density synthetic oligonucleotide arrays. *Nat. Genet.* **21**, 20-24.

Lisbin, M. J., Qiu, J. and White, K. (2001). The neuron-specific RNA-binding protein ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA. *Genes Dev.* **15**, 2546-61.

Llamazares, S., Moreira, A., Tavares, A., Girdham, C., Spruce, B. A., Gonzalez, C., Karess, R. E., Glover, D. M. and Sunkel, C. E. (1991). *polo* encodes a protein kinase homolog required for mitosis in *Drosophila*. *Genes Dev.* **5**, 2153-2165.

Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. et al. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* **14**, 1675-1680.

Long, A. R., Wilkins, J. C. and Shepherd, D. (1998). Dynamic developmental expression of *smallminded*, a *Drosophila* gene required for cell division. *Mech. Dev.* **76**, 33-43.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.

Lundell, M. J. and Hirsh, J. (1992). The *zfh-2* gene product is a potential regulator of neuron-specific dopa decarboxylase gene expression in *Drosophila*. *Dev. Biol.* **154**, 84-94.

Luzzi, V., Mahadevappa, M., Raja, R., Warrington, J. A. and Watson, M. A. (2003). Accurate and reproducible gene expression profiles from laser capture microdissection, transcript amplification, and high density oligonucleotide microarray analysis. *J. Mol. Diagn.* **5**, 9-14.

Malatesta, P., Hartfuss, E. and Gotz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-5263.

Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., Philp, A. V., Yang, M., Glover, D., Kaiser, K., Palter, K. and Selleck, S. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of *Drosophila*. *Dev. Dyn.* **209**, 310-322.

Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). *dachshund* encodes a nuclear protein required for normal eye and leg development in *Drosophila*. *Development* **120**, 3473-3486.

Matsuzaki, M. and Saigo, K. (1996). *hedgehog* signaling independent of *engrailed* and *wingless* required for post-S1 neuroblast formation in *Drosophila* CNS. *Development* **122**, 3567-3575.

Maurange, C. and Gould, A. P. (2005). Brainy but not too brainy: starting and stopping neuroblast divisions in *Drosophila*. *Trends Neurosci.* **28**, 30-36.

Maurel-Zaffran, C., Suzuki, T., Gahmon, G., Treisman, J. E. and Dickson, B. J. (2001). Cell-autonomous and -nonautonomous functions of LAR in R7 photoreceptor axon targeting. *Neuron* **32**, 225-235.

McDonald, J. A. and Doe, C. Q. (1997). Establishing neuroblast-specific gene expression in the *Drosophila* CNS: *huckebein* is activated by Wingless and Hedgehog and repressed by Engrailed and Gooseberry. *Development* **124**, 1079-1087.

McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the *Drosophila* central nervous system: the *vnd* homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.

McNeill, H. (2000). Sticking together and sorting things out: adhesion as a force in development. *Nat. Rev. Genet.* **1**, 100-108.

Meinertzhagen, I. A. and Hanson, T. E. (1993). The development of the optic lobe. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1363-1491. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Metin, C. and Godement, P. (1996). The ganglionic eminence may be an intermediate target for corticofugal and thalamocortical axons. *J. Neurosci.* **16**, 3219-3235.

Micchelli, C. A. and Blair, S. S. (1999). Drosoventral lineage restriction in wing imaginal discs requires Notch. *Nature* **401**, 473-476.

Michaut, L., Flister, S., Neeb, M., White, K. P., Certa, U. and Gehring, W. J. (2003). Analysis of the eye developmental pathway in *Drosophila* using DNA microarrays. *Proc. Natl. Acad. Sci. USA* **100**, 4024-4029.

Miller, A. A., Bernardoni, R. and Giangrande, A. (1998). Positive autoregulation of the glial promoting factor glide/gcm. *Embo J.* **17**, 6316-6326.

Mills, J. C., Roth, K. A., Cagan, R. L. and Gordon, J. I. (2001). DNA microarrays and beyond: completing the journey from tissue to cell. *Nat. Cell Biol.* **3**, E175-178.

Misra, S., Crosby, M., Mungall, C., Matthews, B., Campbell, K., Hradecky, P., Huang, Y., Kaminker, J., Millburn, G., Prochnik, S. et al. (2002). Annotation of the *Drosophila melanogaster* euchromatic genome: a systematic review. *Genome Biol.* **3**, research0083.

Mitrofanis, J. and Baker, G. E. (1993). Development of the thalamic reticular and perireticular nuclei in rats and their relationship to the course of growing corticofugal and corticopetal axons. *J. Comp. Neurol.* **338**, 575-587.

Montalta-He, H. and Reichert, H. (2003). Impressive expressions: developing a systematic database of gene-expression patterns in *Drosophila* embryogenesis. *Genome Biol.* **4**, 205.

Montalta-He, H., Leemans, R., Loop, T., Strahm, M., Certa, U., Primig, M., Acampora, D., Simeone, A. and Reichert, H. (2002). Evolutionary conservation of *otd*/*Otx2* transcription factor action: a genome-wide microarray analysis in *Drosophila*. *Genome Biol.* **3**, RESEARCH0015.

Morante, J. and Desplan, C. (2004). Building a projection map for photoreceptor neurons in the *Drosophila* optic lobes. *Semin. Cell Dev. Biol.* **15**, 137-143.

Morante, J. and Desplan, C. (2005). Photoreceptor axons play hide and seek. *Nat. Neurosci.* **8**, 401-402.

Munro, S. (2003). Lipid rafts: elusive or illusive? *Cell* **115**, 377-388.

Myers, E. W., Sutton, G. G., Delcher, A. L., Dew, I. M., Fasulo, D. P., Flanigan, M. J., Kravitz, S. A., Mobarry, C. M., Reinert, K. H., Remington, K. A. et al. (2000). A whole-genome assembly of *Drosophila*. *Science* **287**, 2196-2204.

Nassif, C., Noveen, A. and Hartenstein, V. (2003). Early development of the *Drosophila* brain: III. The pattern of neuropile founder tracts during the larval period. *J. Comp. Neurol.* **455**, 417-434.

Neel, V. A. and Young, M. W. (1994). *Igloo*, a GAP-43-related gene expressed in the developing nervous system of *Drosophila*. *Development* **120**, 2235-2243.

Newsome, T. P., Asling, B. and Dickson, B. J. (2000). Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-860.

Ng, S. C., Perkins, L. A., Conboy, G., Perrimon, N. and Fishman, M. C. (1989). A *Drosophila* gene expressed in the embryonic CNS shares one conserved domain with the mammalian GAP-43. *Development* **105**, 629-638.

Nigg, E. A. (1998). Polo-like kinases: positive regulators of cell division from start to finish. *Curr. Opin. Cell Biol.* **10**, 776-783.

Noordermeer, J. N., Kopczynski, C. C., Fetter, R. D., Bland, K. S., Chen, W. Y. and Goodman, C. S. (1998). Wrapper, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheath commissural axons in *Drosophila*. *Neuron* **21**, 991-1001.

Novotny, T., Eiselt, R. and Urban, J. (2002). Hunchback is required for the specification of the early sublineage of neuroblast 7-3 in the *Drosophila* central nervous system. *Development* **129**, 1027-1036.

O'Connor-Giles, K. M. and Skeath, J. B. (2003). Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in *Drosophila*. *Dev. Cell* **5**, 231-243.

Ohno, K., Hirose, F., Inoue, Y. H., Takisawa, H., Mimura, S., Hashimoto, Y., Kiyono, T., Nishida, Y. and Matsukage, A. (1998). cDNA cloning and expression during development of *Drosophila melanogaster* MCM3, MCM6 and MCM7. *Gene* **217**, 177-185.

Ohnuma, S., Philpott, A. and Harris, W. A. (2001). Cell cycle and cell fate in the nervous system. *Curr. Opin. Neurobiol.* **11**, 66-73.

Oishi, I., Sugiyama, S., Liu, Z. J., Yamamura, H., Nishida, Y. and Minami, Y. (1997). A novel *Drosophila* receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. *J. Biol. Chem.* **272**, 11916-11923.

Okajima, T. and Irvine, K. D. (2002). Regulation of notch signaling by o-linked fucose. *Cell* **111**, 893-904.

Pearson, A., Lux, A. and Krieger, M. (1995). Expression cloning of *dSR-CI*, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U S A* **92**, 4056-4060.

Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in *Drosophila*. *Nature* **425**, 624-628.

Pereanu, W., Shy, D. and Hartenstein, V. (2005). Morphogenesis and proliferation of the larval brain glia in *Drosophila*. *Dev. Biol.* In press.

Platt, N., da Silva, R. P. and Gordon, S. (1998). Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol* **8**, 365-372.

Poeck, B., Fischer, S., Gunning, D., Zipursky, S. L. and Salecker, I. (2001). Glial cells mediate target layer selection of retinal axons in the developing visual system of *Drosophila*. *Neuron* **29**, 99-113.

Poeck, B., Hofbauer, A. and Pflugfelder, G. O. (1993). Expression of the *Drosophila optomotor-blind* gene transcript in neuronal and glial cells of the developing nervous system. *Development* **117**, 1017-1029.

Prakash, S., Caldwell, J. C., Eberl, D. F. and Clandinin, T. R. (2005). *Drosophila* N-cadherin mediates an attractive interaction between photoreceptor axons and their targets. *Nat. Neurosci.* **8**, 443-450.

Prokopenko, S. N., He, Y., Lu, Y. and Bellen, H. J. (2000). Mutations affecting the development of the peripheral nervous system in *Drosophila*: a molecular screen for novel proteins. *Genetics* **156**, 1691-1715.

Qian, X., Shen, Q., Goderie, S. K., He, W., Capela, A., Davis, A. A. and Temple, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. *Neuron* **28**, 69-80.

Ragone, G., Van De Bor, V., Sorrentino, S., Kammerer, M., Galy, A., Schenck, A., Bernardoni, R., Miller, A. A., Roy, N. and Giangrande, A. (2003). Transcriptional regulation of glial cell specification. *Dev. Biol* **255**, 138-150.

Rajagopalan, D. (2003). A comparison of statistical methods for analysis of high density oligonucleotide array data. *Bioinformatics* **19**, 1469-1476.

Rangarajan, R., Courvoisier, H. and Gaul, U. (2001). Dpp and Hedgehog mediate neuron-glia interactions in *Drosophila* eye development by promoting the proliferation and motility of subretinal glia. *Mech. Dev.* **108**, 93-103.

Rangarajan, R., Gong, Q. and Gaul, U. (1999). Migration and function of glia in the developing *Drosophila* eye. *Development* **126**, 3285-3292.

Rao, Y., Pang, P., Ruan, W., Gunning, D. and Zipursky, S. L. (2000). *brakeless* is required for photoreceptor growth-cone targeting in *Drosophila*. *Proc. Natl. Acad. Sci.* **97**, 5966-5971.

Rauskolb, C., Correia, T. and Irvine, K. D. (1999). Fringe-dependent separation of dorsal and ventral cells in the *Drosophila* wing. *Nature* **401**, 476-480.

Rauson, J. M., Dimitroff, B., Johnson, K. G., Rawson, J. M., Ge, X., Van Vactor, D. and Selleck, S. B. (2005). The heparan sulfate proteoglycans Dally-like and syndecan have distinct functions in axon guidance and visual-system assembly in *Drosophila*. *Curr. Biol.* **15**, 833-838.

Rebay, I. and Rubin, G. M. (1995). Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell* **81**, 857-866.

Reese, M. G., Hartzell, G., Harris, N. L., Ohler, U., Abril, J. F. and Lewis, S. E. (2000). Genome annotation assessment in *Drosophila melanogaster*. *Genome Res.* **10**, 483-501.

Reichert, H. and Simeone, A. (2001). Developmental genetic evidence for a monophyletic origin of the bilaterian brain. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356**, 1533-1544.

Reichert, H. and Boyan, G. (1997). Building a brain: developmental insights in insects. *Trends. Neurosci.* **20**, 258-264.

Rietveld, A., Neutz, S., Simons, K. and Eaton, S. (1999). Association of sterol- and glycosylphosphatidylinositol-linked proteins with *Drosophila* raft lipid microdomains. *J. Biol. Chem.* **274**, 12049-12054.

Roark, M., Sturtevant, M. A., Emery, J., Vaessin, H., Grell, E. and Bier, E. (1995). *scratch*, a pan-neural gene encoding a zinc finger protein related to snail, promotes neuronal development. *Genes Dev.* **9**, 2384-2398.

Robinow, S., Campos, A. R., Yao, K. M. and White, K. (1988). The *elav* gene product of *Drosophila*, required in neurons, has three RNP consensus motifs. *Science* **242**, 1570-1572.

Roegiers, F. and Jan, Y. N. (2004). Asymmetric cell division. *Curr. Opin. Cell Biol.* **16**, 195-205.

Roegiers, F., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (2001). Two types of asymmetric divisions in the *Drosophila* sensory organ precursor cell lineage. *Nat. Cell Biol.* **3**, 58-67.

Roy, P. J., Stuart, J. M., Lund, J. and Kim, S. K. (2002). Chromosomal clustering of muscle-expressed genes in *Caenorhabditis elegans*. *Nature* **418**, 975-979.

Ruan, W., Pang, P. and Rao, Y. (1999). The SH2/SH3 adaptor protein dock interacts with the Ste20-like kinase misshapen in controlling growth cone motility. *Neuron* **24**, 595-605.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Rubsam, R., Hollmann, M., Simmerl, E., Lammermann, U., Schafer, M. A., Buning, J. and Schafer, U. (1998). The *egghead* gene product influences oocyte differentiation by follicle cell-germ cell interactions in *Drosophila* melanogaster. *Mech. Dev.* **72**, 131-140.

Safarik, I. and Safarikova, M. (1999). Use of magnetic techniques for the isolation of cells. *J. Chromatogr. B Biomed. Sci. Appl.* **722**, 33-53.

Salecker, I. and Boeckh, J. (1996). Influence of receptor axons on the formation of olfactory glomeruli in a hemimetabolous insect, the cockroach *Periplaneta americana*. *J. Comp. Neurol.* **370**, 262-279.

Salecker, I., Clandinin, T. R. and Zipursky, S. L. (1998). Hedgehog and Spitz: making a match between photoreceptor axons and their targets. *Cell* **95**, 587-590.

Sanchez-Soriano, N. and Russell, S. (2000). Regulatory mutations of the *Drosophila* Sox gene *Dichaete* reveal new functions in embryonic brain and hindgut development. *Dev. Biol.* **220**, 307-321.

Schena, M., Shalon, D., Davis, R. W. and Brown, P. O. (1995). Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* **270**, 467-470.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of *Drosophila* embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Schmidt, H., Rickert, C., Bossing, T., Vef, O., Urban, J. and Technau, G. M. (1997). The embryonic central nervous system lineages of *Drosophila melanogaster*. II. Neuroblast lineages derived from the dorsal part of the neuroectoderm. *Dev. Biol.* **189**, 186-204.

Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A. (1997). Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. *Development* **124**, 1215-1226.

Schneitz, K., Spielmann, P. and Noll, M. (1993). Molecular genetics of Aristaless, a prd-type homeo box gene involved in the morphogenesis of proximal and distal pattern elements in a subset of appendages in Drosophila. *Genes Dev.* **7**, 114-129.

Scholz, H., Sadlowski, E., Klaes, A. and Klambt, C. (1997). Control of midline glia development in the embryonic *Drosophila* CNS. *Mech. Dev.* **64**, 137-151.

Schreiber, J., Enderich, J. and Wegner, M. (1998). Structural requirements for DNA binding of GCM proteins. *Nucleic. Acids Res.* **26**, 2337-2343.

Schreiber, J., Sock, E. and Wegner, M. (1997). The regulator of early gliogenesis glial cells missing is a transcription factor with a novel type of DNA-binding domain. *Proc. Natl. Acad. Sci. USA* **94**, 4739-4744.

Schultz, S. J., Fry, A. M., Sutterlin, C., Ried, T. and Nigg, E. A. (1994). Cell cycle-dependent expression of *Nek2*, a novel human protein kinase related to the NIMA mitotic regulator of *Aspergillus nidulans*. *Cell Growth Differ.* **5**, 625-635.

Senti, K. A., Keleman, K., Eisenhaber, F. and Dickson, B. J. (2000). *brakeless* is required for lamina targeting of R1-R6 axons in the *Drosophila* visual system. *Development* **127**, 2291-2301.

Senti, K. A., Usui, T., Boucke, K., Greber, U., Uemura, T. and Dickson, B. J. (2003). Flamingo regulates R8 axon-axon and axon-target interactions in the *Drosophila* visual system. *Curr. Biol.* **13**, 828-832.

Shishido, E., Ono, N., Kojima, T. and Saigo, K. (1997). Requirements of DFR1/Heartless, a mesoderm-specific *Drosophila* FGF-receptor, for the formation of heart, visceral and somatic muscles, and ensheathing of longitudinal axon tracts in CNS. *Development* **124**, 2119-2128.

Simons, K. and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31-39.

Skeath, J. B. (1998). The *Drosophila* EGF receptor controls the formation and specification of neuroblasts along the dorsal-ventral axis of the *Drosophila* embryo. *Development* **125**, 3301-3312.

Skeath, J. B. and Carroll, S. B. (1994). The *achaete-scute* complex: generation of cellular pattern and fate within the *Drosophila* nervous system. *Faseb J* **8**, 714-721.

Skeath, J. B. and Thor, S. (2003). Genetic control of *Drosophila* nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.

Skeath, J. B., Zhang, Y., Holmgren, R., Carroll, S. B. and Doe, C. Q. (1995). Specification of neuroblast identity in the *Drosophila* embryonic central nervous system by *gooseberry-distal*. *Nature* **376**, 427-430.

Soller, M. and White, K. (2003). ELAV inhibits 3'-end processing to promote neural splicing of ewg pre-mRNA. *Genes Dev.* **17,** 2526-2538.

Soriano, N. S. and Russell, S. (1998). The *Drosophila* SOX-domain protein Dichaete is required for the development of the central nervous system midline. *Development* **125**, 3989-3996.

Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the *Drosophila* embryo. *Dev. Biol.* **246**, 57-67.

Steffensen, S., Coelho, P. A., Cobbe, N., Vass, S., Costa, M., Hassan, B., Prokopenko, S. N., Bellen, H., Heck, M. M. and Sunkel, C. E. (2001). A role for *Drosophila* SMC4 in the resolution of sister chromatids in mitosis. *Curr. Biol.* **11**, 295-307.

Steigemann, P., Molitor, A., Fellert, S., Jackle, H. and Vorbruggen, G. (2004). Heparan sulfate proteoglycan Syndecan promotes axonal and myotube guidance by Slit/Robo signaling. *Curr. Biol.* **14**, 225-230.

Steward, R. (1987). Dorsal, an embryonic polarity gene in *Drosophila*, is homologous to the vertebrate proto-oncogene, *c-rel*. *Science* **238**, 692-694.

Stivers, C., Brody, T., Kuzin, A. and Odenwald, W. F. (2000). *Nerfin-1* and *-2*, novel *Drosophila* Zn-finger transcription factor genes expressed in the developing nervous system. *Mech. Dev.* **97**,205-210.

Stollewerk A. and Klambt C. (1997). The midline glial cells are required for regionalization of commissural axons in the embryonic CNS of *Drosophila*. *Dev. Genens Evol.* **207**, 401-409.

Stowers, R. S. and Schwarz, T. L. (1999). A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics* **152**, 1631-1639.

Su, Y. C., Maurel-Zaffran, C., Treisman, J. E. and Skolnik, E. Y. (2000). The Ste20 kinase misshapen regulates both photoreceptor axon targeting and dorsal closure, acting downstream of distinct signals. *Mol. Cell Biol.* **20**, 4736-4744.

Suh, G. S., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D. A. and Zipursky, S. L. (2002). *Drosophila* JAB1/CSN5 acts in photoreceptor cells to induce glial cells. *Neuron* **33**, 35-46.

Tautz D. and Pfeifle C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Tayler, T. D. and Garrity, P. A. (2003). Axon targeting in the *Drosophila* visual system. *Curr. Opin. Neurobiol.* **13**, 90-95.

Tayler, T. D., Robichaux, M. B. and Garrity, P. A. (2004). Compartmentalization of visual centers in the Drosophila brain requires Slit and Robo proteins. *Development* **131**, 5935-5945.

Therianos, S., Leuzinger, S., Hirth, F., Goodman, C. S. and Reichert, H. (1995). Embryonic development of the *Drosophila* brain: formation of commissural and descending pathways. *Development* **121**, 3849-3860.

Tietjen, I., Rihel, J. M., Cao, Y., Koentges, G., Zakhary, L. and Dulac, C. (2003). Single-cell transcriptional analysis of neuronal progenitors. *Neuron* **38**, 161-175.

Ting, C. Y., Yonekura, S., Chung, P., Hsu, S., Robertson, H. M., Chiba, A. and Lee, C. H. (2005). *Drosophila* N-cadherin functions in the first stage of the two-stage layer-selection process of R7 photoreceptor afferents. *Development* **132**, 953-963.

Tix, S., Eule, E., Fischbach, K. F. and Benzer, S. (1997). Glia in the chiasms and medulla of the *Drosophila melanogaster* optic lobes. *Cell Tissue Res.* **289**, 397-409.

Tomancak, P., Beaton, A., Weiszmann, R., Kwan, E., Shu, S., Lewis, S. E., Richards, S., Ashburner, M., Hartenstein, V., Celniker, S. E. et al. (2002). Systematic determination of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol.* **3**, RESEARCH0088.

Truman, J. W. and Bate, M. (1988). Spatial and temporal patterns of neurogenesis in the central nervous system of *Drosophila melanogaster*. *Dev. Biol.* **125**, 145-157.

Tsui-Pierchala, B. A., Encinas, M., Milbrandt, J. and Johnson, E. M., Jr. (2002). Lipid rafts in neuronal signaling and function. *Trends Neurosci.* **25**, 412-417.

Udolph, G., Rath, P. and Chia, W. (2001). A requirement for Notch in the genesis of a subset of glial cells in the *Drosophila* embryonic central nervous system which arise through asymmetric divisions. *Development* **128**, 1457-1466.

Umesono, Y., Hiromi, Y. and Hotta, Y. (2002). Context-dependent utilization of Notch activity in *Drosophila* glial determination. *Development* **129**, 2391-2399.

Van De Bor, V. and Giangrande, A. (2001). Notch signaling represses the glial fate in fly PNS. *Development* **128**, 1381-1390.

Vincent, S., Vonesch, J. L. and Giangrande, A. (1996). Glide directs glial fate commitment and cell fate switch between neurones and glia. *Development* **122**, 131-139.

Von Ohlen, T. and Doe, C. Q. (2000). Convergence of *dorsal*, *dpp*, and *egfr* signaling pathways subdivides the *Drosophila* neuroectoderm into three dorsal-ventral columns. *Dev. Biol.* **224**, 362-372.

Wandall, H. H., Pedersen, J. W., Park, C., Levery, S. B., Pizette, S., Cohen, S. M., Schwientek, T. and Clausen, H. (2003). *Drosophila egghead* encodes a beta 1,4-mannosyltransferase predicted to form the immediate precursor glycosphingolipid substrate for brainiac. *J. Biol. Chem.* **278**, 1411-1414.

Wandall, H. H., Pizette, S., Pedersen, J. W., Eichert, H., Levery, S. B., Mandel, U., Cohen, S. M. and Clausen, H. (2005). Egghead and Brainiac are essential for glycosphingolipid biosynthesis in vivo. *J. Biol. Chem.* **280**, 4858-4863.

Watts, R. W. (2003). A historical perspective of the glycosphingolipids and sphingolipidoses. *Philos. Trans. R. Soc. Lond.* B *Biol. Sci.* **358**, 975-983.

Wech, I., Bray, S., Delidakis, C. and Preiss, A. (1999). Distinct expression patterns of different enhancer of split bHLH genes during embryogenesis of *Drosophila melanogaster*. *Dev. Genes Evol.* **209**, 370-375.

Wegner, M. and Riethmacher, D. (2001). Chronicles of a switch hunt: gcm genes in development. *Trends Genet.* **17**, 286-290.

Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the *Drosophila* central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.

Wharton, K. A., Ray, R. P. and Gelbart, W. M. (1993). An activity gradient of *decapentaplegic* is necessary for the specification of dorsal pattern elements in the *Drosophila* embryo. *Development* **117**, 807-822.

Williams, B. C., Riedy, M. F., Williams, E. V., Gatti, M. and Goldberg, M. L. (1995). The *Drosophila* kinesin-like protein KLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. *J. Cell Biol.* **129**, 709-723.

Winberg, M. L., Perez, S. E. and Steller, H. (1992). Generation and early differentiation of glial cells and their progenitors in the *Drosophila* embryo. *Development* **127**, 4959-4969.

Wolff, T. and Ready, D. F. (1993). Pattern formation in the Drosophila retina. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1277-1325. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Xiong, W. C. and Montell, C. (1995). Defective glia induce neuronal apoptosis in the *repo* visual system of *Drosophila*. *Neuron* **14**, 581-590.

Xiong, W. C., Okano, H., Patel, N. H., Blendy, J. A. and Montell, C. (1994). *repo* encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. *Genes Dev.* **8**, 981-994.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

Yagi, Y., Suzuki, T. and Hayashi, S. (1998). Interaction between *Drosophila* EGF receptor and *vnd* determines three dorsoventral domains of the neuroectoderm. *Development* **125**, 3625-3633.

Yang, H. and Kunes, S. (2004) Nonvesicular release of acetylcholine is required for axon targeting in the *Drosophila* visual system. *Proc. Natl. Acad. Sci.* **101**, 15213-15218.

Yang, Y. H. and Speed, T. (2002). Design issues for cDNA microarray experiments. *Nat. Rev. Genet.* **3**, 579-588.

Yao, K. M., Samson, M. L., Reeves, R. and White, K. (1993). Gene *elav* of *Drosophila melanogaster*: a prototype for neuronal-specific RNA binding protein gene family that is conserved in flies and humans. *J. Neurobiol.* **24**, 723-739.

Younossi-Hartenstein, A., Salvaterra, P. M. and Hartenstein, V. (2003). Early development of the *Drosophila* brain: IV. Larval neuropile compartments defined by glial septa. *J. Comp. Neurol.* **455**, 435-450.

Yuasa, Y., Okabe, M., Yoshikawa, S., Tabuchi, K., Xiong, W. C., Hiromi, Y. and Okano, H. (2003). *Drosophila* homeodomain protein REPO controls glial differentiation by cooperating with ETS and BTB transcription factors. *Development* **130**, 2419-2428.

Zecca, M. and Struhl, G. (2002). Control of growth and patterning of the Drosophila wing imaginal disc by EGFR-mediated signaling. *Development* **129**, 1369-1376.

Zeltser, L. M., Larsen, C. W. and Lumsden, A. (2001). A new developmental compartment in the forebrain regulated by Lunatic fringe. *Nat. Neurosci.* **4**, 683-684.

Zhang, Y., Ungar, A., Fresquez, C. and Holmgren, R. (1994). Ectopic expression of either the *Drosophila gooseberry-distal* or *proximal* gene causes alterations of cell fate in the epidermis and central nervous system. *Development* **120**, 1151-1161.

Zilian, O., Frei, E., Burke, R., Brentrup, D., Gutjahr, T., Bryant, P. J. and Noll, M. (1999). *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. *Development* **126**, 5409-5420.

Zipursky, S. L., Venkatesh, T. R., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

ACKNOWLEDGEMENTS

I'm very grateful to my supervisor, Prof. Dr. Heinrich Reichert, for introducing me into the neurobiology of *Drosophila* and for helping me in many aspects of my scientific career. Many thanks go as well to Prof. Dr. Karl-Friedrich Fischbach and to Prof. Dr. Silvia Arber for serving in my thesis committee.

I'm very grateful to my friends and colleagues in the Reichert lab, the Institute of Zoology and the Biocenter.

My very special thanks go to my wife Na Qu and to our families for their continuous support during the time of my Ph.D. study.

ERKLÄRUNG

Ich erkläre, dass ich die Dissertation

NEUROGLIOGENESIS AND VISUAL SYSTEM DEVELOPMENT IN *DROSOPHILA*: GENETIC/ GENOMIC ANALYSIS OF THE *GLIAL CELLS MISSING* AND *EGGHEAD* GENES

nur mit der darin angegebenen Hilfe verfasst und bei keiner anderen Universität und keiner anderen Fakultät der Universität Basel eingereicht habe.

Basel, den 10. Juni 2005

Yun Fan